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C.-E. A. WINSLOW

It is characteristic of Science and Progress that they continually open new fields to our vision.—PASTEUR.

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METHOD FOR THE ISOLATION OF BACTERIA IN PURE CULTURE FROM SINGLE CELLS AND PROCEDURE FOR THE DIRECT TRACING OF BACTERIAL GROWTH ON A SOLID MEDIUM

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By a pure culture we understand, as is well-known, a culture consisting of individuals of which we know with certainty that all are descended from one single cell, and from one only. As all bacteriologic work, of whatever kind it may be, depends on our working with such reliable pure cultures, many efforts have, of course, been made in the course of time in order to devise reliable methods of isolating a single bacterium.

The first investigator who solved the problem in a satisfactory way,—although not in regard to the bacteria proper—was Emil Chr. Hansen. The principle of his method was, briefly stated, to observe directly under the microscope the growth of the individual yeast-cell until it has formed a small colony in a gelatin droplet on the lower surface of a coverglass in a moist chamber. Yeast-cells are however far bigger than most bacteria, and there is no possibility of tracing with any certainty the growth of a bacterium, as for instance a colon bacillus, in a similar way in gelatin.

Of methods that have been proposed and employed for single cell cultivation of bacteria, the best known are those of Schouten, Barber and Malone, none of which have however attained any extensive application, no doubt partly owing to the intricate apparatus they require, and partly to the difficulty involved in picking up such minute objects as bacteria with such relatively coarse implements as pipettes and loops; and when Barber states that he is able to pick up successively each single one of four

bacteria, which he views in a small hanging-drop of broth, and to inoculate four broth test tubes with each of them separately, one feels predisposed to doubt the possibility of ever acquiring such practice.

The method most generally used at present is, no doubt, Burri's India ink method, which is the starting point for the procedure described in the following communication. As is well known, the principles of the India ink method are, briefly stated, these: the bacteria are emulsified in diluted India ink, of which emulsion minute droplets are deposited on a gelatin plate in a Petri dish by means of a mapping-pen. Those droplets which, by microscopical investigation with a high power dry lens, prove to contain only one single cell, are noted and allowed to stand until a small colony has developed, from which subcultures are prepared; or, the India ink droplet is removed, together with the bacterium, by means of a sterile coverslip that is superimposed on the black spot of the gelatin plate, removed again together with the India ink and the bacterium, and dropped into an appropriate fluid nutrient medium. This is, as has been said, an excellent method, by means of which, with some practice and patience, reliable single-cell cultures of most species of bacteria can fairly easily be produced. (It is, however, not all bacterial species that will stand the India ink.)

A drawback in Burri's method is the necessity of having the unhandy Petri dish standing on the microscope stage during the examination. Therefore, I devised a modification: by means of sterile Pasteur pipettes I poured liquid gelatin upon previously sterilized slides. On the gelatin surface three rows of India ink droplets were deposited, which could now be much more easily and rapidly examined by shifting the mechanical stage, the selected India ink droplets being subsequently removed as usual by means of sterile coverslips.

If it is desired to trace the development of the new formed elements on the gelatin, the slides are placed in a sterile Petri dish with a piece of moist filter paper at the bottom. In this way the India ink spot can be examined at intervals, and the development can be observed. The image however, will rapidly

become blurred under such conditions. The India ink will be broken up, the new-formed elements pushing in beneath it; even though the India ink be highly diluted, which facilitates observation, the image will rapidly lose its sharpness. Our objective being, in my case, partly to obtain single-cell cultures from some atypical bacterial elements, and partly to trace their development, it seemed natural, to attempt to do completely without the India ink, the multiplication of the bacteria being much easier to follow, the more dilute the India ink. When this was done I admit being surprised at seeing how well the organisms showed up as sharply defined, highly refractive elements, readily distinguishable from other chance particles or impurities on the surface.

Gelatin is however not a particularly suitable medium for most bacteria, and growth at 37°C. could not be observed in this manner, so I tried whether the bacteria were as clearly visible on an agar surface. In order to study this point, agar (common filtered broth agar) was melted, and by means of a coarse Pasteur pipette poured over a sterile slide. The bacteria were even more readily discernible on this surface than on the gelatin. It is difficult however to procure a perfectly level agar surface in this manner. This difficulty was overcome by abandoning the pouring of the agar on the slide and, instead, excising the medium out of an agar plate in a Petri dish by means of a knife, previously sterilized in a flame and cooled, lifting the excised cube of agar on the blade of the knife and depositing it on the sterilized slide to which it will immediately adhere.

The essential conditions were now provided for tracing bacterial growth on a solid medium, especially if certain difficulties could be overcome in regard to ensuring reliable and readily obtained single-cell cultures. At this point in my investigation a paper appeared in The Journal of Hygiene by Hort, in which he describes a method, the underlying principles of which are the same, namely, the observation of unstained bacteria without a contrast on an agar surface, partly by means of oil-immersion lenses, partly by a system of high-power dry lenses. Having given a review of the usually employed methods of isolation,

all of which, including Burri's method, he rejects, he suggests a new method of isolation having two modifications, one in which examination is undertaken with an oil-immersion lens, and one in which a dry lens is employed.

In bacteriologic literature, Hort is thus the first to point out that it is possible to see bacteria distinctly on the surface of an agar plate and to watch their growth by means of a high-power system of dry lenses without any staining or contrast. It is true that Hill, in his work on the morphology of the diphtheria bacillus, mentions that diphtheria bacilli can be seen distinctly on agar by means of a high-power dry lens, but he does not seem to realize the possibilities involved in this fact. It is no doubt possible, and I think, probable, that others too have been aware of this fact; Hort is however, as stated, the first who has defined it and understood how to make use of it.

The medium which Hort employs in his examination, he prepares in a manner similar to the one originally applied by me. He pours the hot agar over sterile slides in as level a layer as possible, taking care to keep the agar well within the edges of the slide. The mode of procedure now varies as to whether he employs the oil-immersion lens, or the dry lens system for further examination. In the first case a series of sterile coverslips have been previously prepared, each with a small circle etched on its surface by means of a diamond. In the center of this circle, a minute droplet of broth is now deposited, taken from a broth culture containing the bacteria under investigation in a suitable emulsion. The inoculated coverslip is now placed face downwards on the agar surface; the area within the small circle is thoroughly examined with an oil-immersion lens, and, in case only one single organism is found within the circle, the slide is placed in a Petri dish which is then placed in the incubator. The circle is examined at short intervals, until a small colony has formed from which subcultures can be prepared.

Hort himself remarks, in regard to this procedure, that great care must be taken to ensure that the droplet does not run outside the etched circle when the coverslip is applied to the agar, adding, however, that with some care this is easily avoided. It

is evident that we must feel perfectly sure on this point, considering that the area within the circle is the only one examined. However, it seems difficult to understand how one can be sure that the small droplet keeps within the etched circle, as a small liquid layer will form between the coverslip and the agar, the moment these two are directly applied to each other, which will immediately make the droplet invisible. The possibility will likewise always exist, of various currents arising between coverslip and agar, both when the slip is placed and removed; and, even though the growth of a colony from one single cell has been observed within the circle, this may become contaminated from a small colony immediately outside the circle, the moment the coverslip is removed. As a rule, there is little chance of this happening, but it does compromise the reliability of the method.

Moreover, there is the question of the power of certain motile bacteria to move on the uncovered agar plate. The possibility of such active motility on the part of the organisms is, of course, increased by the placing of the coverslip on the agar, by which a small liquid-filled space is formed.

For his second method Hort employs a medium prepared in a similar way. A highly diluted emulsion of bacteria is spread over the agar plate by means of a glass rod, the inoculated plate being now covered with a thin sterile strip of celluloid, which has been previously perforated. Small sterile coverslips are placed over the holes so as to form minute moist chambers. These are now searched with a high-power dry lens and the chambers containing one single cell only, are marked. The examination is now continued as described above. (It is not evident from Hort's treatise, whether the coverslips are removed during the examination under the microscope; if not, one would think that the dew on the coverslip would be obstructive.) The colonies having reached an appropriate size, subcultures are prepared by means of a special apparatus consisting of a tube with a needle adjusted in a special way and screwed on to the nose-piece like an objective. This method is reliable, but as Hort himself says in his final remarks: "In conclusion it is necessary to point out that cultivation of bacteria from single cells is, even when

employing a good method, a most tedious procedure, involving several hours' close work for each organism isolated, if the results are to be relied on."

I believe the difficulties to have become considerably reduced in the procedure described in the following pages, and, the simpler a method is, the more reliable will it generally be. The principle upon which the method of isolation described below has been based is, as mentioned, the fact that a bacterium—possibly the minutest ones excepted—can readily be distinguished on the surface of a clear transparent medium, such as for instance agar, gelatin, or ascitic agar. The mode of procedure is, briefly stated, as follows: A young bacterial culture, such for instance as a twelve-hour old broth culture of colon bacilli, is inoculated on the agar plate in a Petri dish. The agar had better not be more than a few millimeters thick (bacteria can also be distinguished on very thick agar, but less sharply). The upper and lower surfaces of the agar should be parallel so as to ensure that the excised bits shall be of equal thickness everywhere, partly in order to obtain plane images, partly to avoid the risk of running down with the objective into the agar, which is in its immediate vicinity during examination.

It is important, in inoculating the culture, to be fairly clear at the outset as to the density of bacteria desired on the plate. In the diagram, figure 1, some dotted lines show how I am accustomed to proceed. A big droplet of the broth culture is deposited in the center of the circle, and, by means of a glass rod, bent at a right angle, the drop is pressed down between the parallel dotted lines. Now the glass rod is moved from side to side across the first inoculated area, and, finally, the remaining half of the dish is inoculated and it is placed in the incubator for about one hour at 37°C. (Inoculation can of course also be performed in a streak, which some will perhaps find more to the purpose, and in this way an appropriate difference in the density of the bacteria can likewise be obtained.) This measure is taken because the development in the case of the colon bacillus begins just after the expiration of one hour, and because bacteria are more readily discernible when in development, owing

to their increased refractive power. As previously described, a suitable square of the agar is now excised and placed upon the previously sterilized microscope slide, which is most conviently sterilized by flaming (cf. fig. 2).

The microscope slide plus agar, which, for convenience sake, I shall term "slide" in the following discussion, is placed on the stage of the microscope, and an area is chosen where the organisms are placed at a convenient mutual distance, commencing

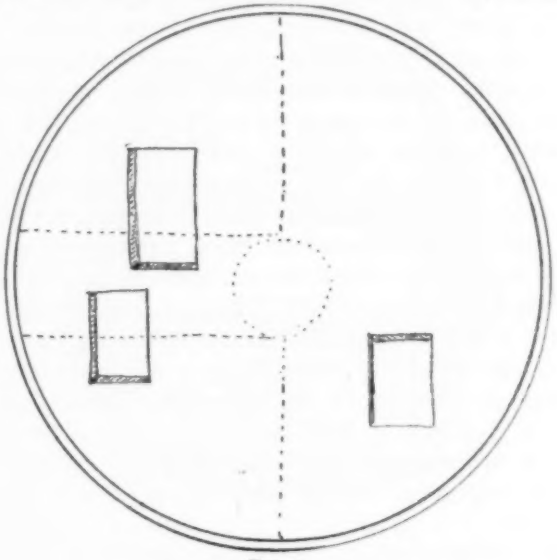

FIG. 1

the examination where they are lying close, and thence, by means of the mechanical stage, proceeding to where they are lying more scattered.[1]

Having now come upon an area where there is one organism, only, within the field of vision, and this single bacterium having

[1] The objective exployed by me was a Zeiss Apochromat; any sufficiently powerful objective will however do. The magnification, at which I usually worked, was 750 diameters. Illumination is a very important factor. The source of light must be uniform. I used a powerful metal filament lamp with frosted bulb, the light of which was considerably reduced by means of the diaphragm of the illuminating apparatus of the microscope.

been centered, the area is noted by means of the mutual relation of the regular scale and the vernier attached to the mechanical stage, and one should now be able to focus exactly in the same place again. At this point there is however a great difficulty to be overcome, as the least inaccuracy in the re-adjustment may have the effect of causing the selected organism to vanish from the field of vision, others being substituted, and,

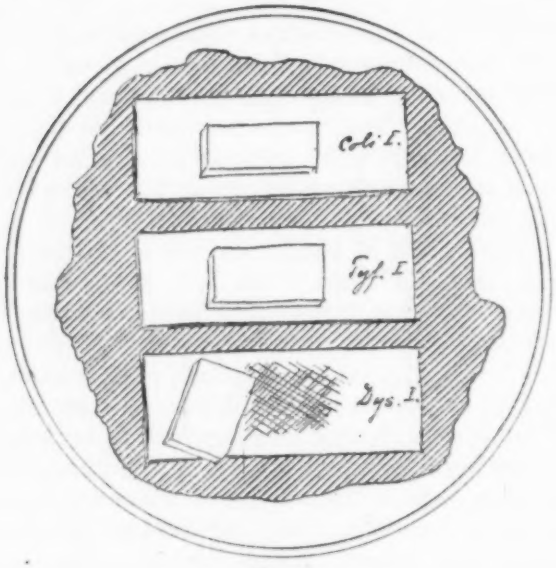

FIG. 2

even though the most painful care be taken, the same fatal accident may happen owing to quite negligible displacements of the scales, which it is often quite impossible to control.

In order to be able to find a particular bacterium again, I have proceeded in the following manner: Prior to placing the agar plate on the slide, a complex of fine lines are by means of a diamond scratched criss-cross, preferably on the lower surface of the slide, over the area to be covered by the agar (cf. fig. 2, above). The lines will become less frayed in the glass if the

scratching be performed in a drop of immersion-oil. In an objective (preferably a different one from that with which the growth is observed, as the micrometer lines will disturb observation) is placed a squared eyepiece micrometer, which is cemented on to prevent displacement.

If we now focus sharply on the scratches on the slide with the low power of the microscope, the lines in the eyepiece micrometer will be intersected by these scratches in a quite specific manner (cf. fig. 3), and thus we obtain two distinguishing marks instead of one. The course of procedure will now be as follows: the agar is placed on the scratched area of the slide, and we search for a place where there is only one organism within the field of vision. The spot is marked by means of the scales of the mechanical stage, the objective with the attached micrometer is placed in the tube, the microscope is adjusted to low magnification and focussed sharply on the scratches. Careful drawings are made on squared paper of the position of these scratches in relation to the eyepiece micrometer, the slide being now placed in a sterile Petri dish with a piece of moist filter paper at the bottom. (The filter paper must not be too wet as this may cause the development of so much aqueous vapour during incubation that the glasses become wet enough for the agar squares to slide, when the whole experiment is ruined.) By means of thus marking the position of the organism we have always succeeded in hitting upon exactly the same spot for repeated examination.

The growth is now watched at proper intervals, the adjustment being performed so that, firstly, the scales of the mechanical stage are placed in the proper mutual relation, which we have noted down, secondly, with the low power of the microscope we make the scratches on the slide correspond to the proper points in the eyepiece micrometer, and, finally, eyepiece and objective are changed, and we can now easily observe the alterations in the small colony in development. (The slides must of course previously be cooled to the same temperature as that of the objective, in case examination takes place at a lower temperature than that of incubation, as, otherwise, condensed moisture

will gather on the front lens and obstruct vision altogether.) In this way, the agar will preserve its shape for at least twenty-four hours, even though subjected to several examinations, provided these be not of too long duration.

Now, do not these repeated examinations involve a great danger of contamination from the air? A risk there is, of course, but it is apparently, insignificant. In the first place it can easily be ascertained that only very few "alien" colonies will be seen

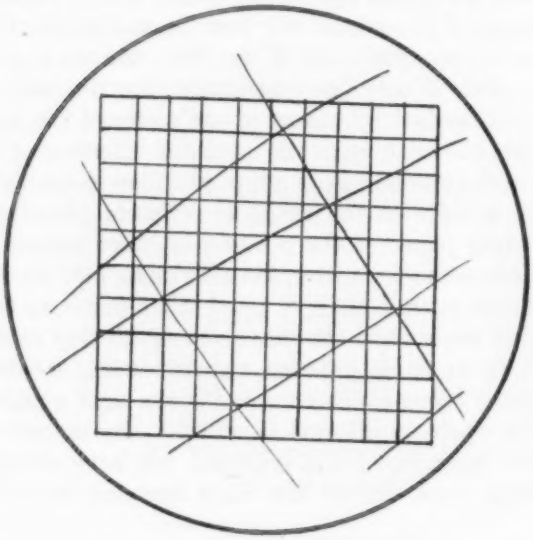

FIG. 3

FIG. 4

to develop in the Petri dish by contamination from the air, in spite of repeated examinations within forty-eight hours, and it would be a stroke of very bad fortune if such a germ from the air should settle just within the field of vision to be examined. If this should occur, it would soon be discovered; it could only escape detection in case a germ from the air dropped upon the selected colony immediately before inoculation was undertaken from it, and then it would most probably be disclosed in further investigations of the bacterial species in question, as it would

be almost inconceivable ill-luck if the "alien" microörganism should be one that was closely related to the isolated one.

Now, the new-formed colony having reached a convenient size, sub-cultures should be prepared from it. At this point the colonies have grown so large as to be distinctly visible with the low power of the microscope, the time and mode of re-inoculation depending on the relative situation of the colonies. In case the colony, the shape of which we recognize with the low power of the microscope, is placed in a sufficiently isolated position, we may defer inoculation until it has grown big enough to allow of our conveniently inoculating from it by means of a fine inoculation needle under the microscope at low magnification. If there is any danger of neighbouring colonies impinging upon it, we must undertake inoculation while it is yet small. As mentioned, Hort used a special apparatus for this purpose. A small harpoon, which one may prepare oneself, will however do.

On the front lens of an objective is placed a small lump of modelling wax to which is attached a fine thin platinum wire not thicker than 0.15 mm. with a blunt end (cf. fig. 4). Previous to "harpooning" the colony some preparatory practice is necessary. A small agar square is excised and fitted in the usual way and placed on a slide. A droplet of India ink or some other staining fluid is deposited on the agar with a mapping-pen. This spot is now centered in the field of vision at the low power of the microscope, and the objective with the attached platinum needle, which is screwed on to the nose-piece, is directed across the spot, the needle being adjusted by a pressure from the fingers so as to be mounted exactly above the spot, and gently depressed so as to touch the agar. The point of contact is readily discerned with the low power of the microscope and marked by means of the eyepiece micrometer. We know now exactly where the needle will hit, being able then to inoculate from the colony by adjusting it to the exact point in relation to the eyepiece micrometer at which the needle hit last. The needle is carefully lowered into the colony until it touches the agar plate. The point of contact is most readily noticed by

following the reflection of the needle in the agar; when the needle and its image meet, contact with the colony has been established. The point of the needle is now touched with broth in a small loop which is raised so as to encompass it several times, agar is inoculated from the loop, and finally the needle is washed in a tube of broth.

The "harpoon" is sterilized by flaming, and now it only remains to examine the area where the colony was previously situated. The bacteria from the colony will be seen to have been scattered somewhat, and we note whether the adjacent colonies are totally intact, both with the low and with the high power of the microscope. In case growth results from the inoculation we know that we have obtained a reliable pure culture. Compared with Hort's dry-lens method, the procedure described presents several advantages. Firstly, it is difficult to pour agar over the slides so as to obtain an even layer, and it takes a long time. Petri dishes with agar are always at hand in any bacteriologic laboratory; these should however be freshly poured to avoid the risk of obtaining pure cultures from chance microörganisms from the air which, being overlooked at inoculation, may have formed small colonies. Secondly, we avoid the perforated celluloid plate which is a hindrance to free operation and means a considerable limitation in the possibility of finding conveniently placed microörganisms. While Hort spends several hours on the pure cultivation of a bacterium by his method, I believe that the total work in my isolation method will, in most cases, only amount to a fraction of an hour for each single bacterium.

As has been shown, we are able to trace the growth from the single cell until a small colony has developed. Details can of course only be observed as long as the colonies are small and single-layered; so soon as the colonies are crowded in several layers, exact examination is of course out of the question. If, for instance, we desire to ascertain whether a morphologically atypical element is viable, and to follow its development, we isolate it as described above and observe its growth at proper intervals. If it is desired to get a survey of the way in which colony formation proceeds we need only, at proper intervals,

to excise small bits of the agar plate inoculated with a bacterial culture, and we obtain in this way a far better picture of the actual morphology of the bacterium than by producing preparations according to the usual methods, whether it be the milder procedure of emulsifying the bacteria in a drop of a staining fluid, or one of the various staining methods with previous fixation.

If, owing to the minuteness or too crowded placing of the microörganisms, we should fail in distinguishing what we desired to see, such as for instance the cell division lines, by means of the dry lens system, we need only place a coverglass on the surface of the agar. This will immediately adhere to the agar, and, by means of the oil-immersion lens we shall be able to detect the bacteria quite distinctly and also to trace, the growth of a single element.

Any one can readily be persuaded as to the facility with which bacteria are distinguished on an agar surface without staining or contrast, by examining an inoculated agar plate after a few hours' incubation.

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BACTERIAL AUTOLYSIS

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The term "autolysis" has quite generally been used to designate a process of cell or tissue deterioration in which the more complex nitrogenous substances, particularly proteins, are reduced to simpler form through the agency of enzymes which have been elaborated by the cells or tissues themselves.

The self-digestion of yeast, and of liver and other animal organs was recognized by Salkowski (1891) to be due to enzymatic action. An increase in the soluble nitrogenous constituents and the presence of leucine and tyrosine were observed. Jacoby (1900) showed that there is a consistent increase in ammonia during the process and advanced the hypothesis that this constituted the mechanism for the elimination of soluble katabolic products of the normal living cell.

Fermi (1890, 1891 and 1894) was among the first to demonstrate the presence of enzymes in bacteria. Extensive comparative studies with pepsin and trypsin showed that the proteolytic principles of bacteria resembled the latter. Our knowledge of the proteolytic enzymes of bacteria was further advanced by Wood (1890), MacFadyen (1892), Vignal (1896), Eijkmann (1901) and Cacace (1901).

By the employment of Buchner's "Hefepressaft" method Hahn (1900) obtained an extract from *Bact. typhosum* and *Mycobact. tuberculosis* which he claimed possessed autolytic properties. Brieger and Mayer (1903 and 1904) reported that they had removed agglutinogens and other specific bodies from *Bact. typhosum* by autolysis. It appears quite probable, however, that the processes which they employed were such

that osmosis cannot be excluded as an important factor. Conradi (1903) described an "autolysis" of *Bact. typhosum* and *Bact. dysenteriae* whereby endotoxins were liberated. He believed that all bacteria possess autolytic enzymes. Neisser and Shiga (1903) reported similar observations. They found, however, that the sterile filtrates of twenty-four hour cultures which had been heated at 60°C. for one hour and kept at 37° for two days showed the presence of free "receptors" which had the power of absorbing agglutinins and depressing agglutination. The filtrate from the dysentery bacillus culture was as toxic as that obtained by Conradi. Hence, in view of this heating, one is not justified in saying that the toxin is liberated by autolysis.

The autolysis of *B. anthracis* was described by Levy and Phersdorff (1902). Microscopic changes were noted in alkaline suspensions to which toluol had been added. Gelatin-liquefying and other enzymes were observed, and the authors claimed that the autolysate was toxic to white mice. However, as much as one thirteenth of the body weight had to be injected in order to obtain these toxicity results.

Rettger (1904) applied chemical criteria to the study of the autolysis of microörganisms and pointed out qualitative changes of considerable significance. He noted the liberation of coagulable protein and subsequently the formation of leucine and tyrosine in water suspensions of *Erythrobacillus prodigiosus*. The biuret test was found to be very valuable in following the course of autolysis. Microscopic changes in stained mounts were followed also. During the incubation of water suspensions of *Bact. coli* to which toluol had been added there was apparently some liberation of coagulable proteins from the cells. It is impossible to state whether this was the result of real autolysis or of osmotic changes.

In Flexner's study of the meningococcus (1907) the interesting observation was made that this organism resists disintegration longer when kept at 37°C. than in the refrigerator. Concentrated suspensions in physiological saline solution were found by microscopic examination to undergo rapid autolysis. Wollstein (1907) showed that the meningococcus and gonococcus

were very much alike in their behavior under the various experimental conditions.

Bürgers, Schermann and Schreiber (1911) claim to have observed autolysis by *Bact. coli*, *Bact. typhosum* and the pneumococcus after killing with chloroform. According to them, however, *B. megatherium*, Staphylococcus and Streptococcus do not autolyze. McClintock and Clark (1909) satisfied themselves that the rapid lysis which suspensions of the gonococcus undergo is due to enzymatic activities. Heating at 70°C. for one hour prevented the cellular changes from taking place. Rosenow (1912) maintained that substances capable of causing anaphylaxis are liberated by the pneumococcus, streptococcus, gonococcus, meningococcus, *Bact. coli*, *Bact. typhosum*, and to some extent by the staphylococcus.

Warden (1913, 1915, and 1917) made a rather extensive study of the autolysis of the gonococcus. In his earlier work he looked in vain for any external factors which might be responsible for the disintegration of the cells, but did observe an "enzyme," tryptic in nature, which was always present in autolyzing suspensions. In a later paper (1915), however, he concludes that "lysis of gonococci. . . . is probably due, not to activities of enzymes, but to other causes, among which water permeability and solution of fatty substances play an important part."

Alilaire (1913), Nicolle (1913) and Salimbeni (1913) studied the process of so-called "autolysis" by determining the "soluble" and "insoluble nitrogen." They claim to have observed increases in "soluble nitrogen" during the autolysis of *Bact. coli*, *Bact. typhosum*, *Proteus vulgaris*, *Pseudomonas pyocyanea*, the gonococcus, meningococcus and pneumococcus. Corper (1916) and Corper and Sweeny (1918) demonstrated that suspensions of the tubercle bacillus in physiological saline solution, with or without the addition of antiseptics, undergo autolysis at 37°C., as evidenced by marked increases in non-coagulable and amino nitrogen.

Dernby (1917 and 1918) working with yeast, and later with animal tissues, was able to show the presence of various proteolytic enzymes having specific hydrogen ion concentration re-

quirements. A pepsin-like enzyme was observed whose activity depended upon a hydrogen ion concentration greater than pH 5.5. In every instance there were also present enzymes of a tryptic nature, most active in a fairly alkaline medium. The acid end of the range of the latter, however, overlapped the alkaline end of the range of the former ferments and a digestion (or autolysis) carried on in the pH zone common to both tended to be much more complete than one carried out in the alkaline range so often chosen for such experiments.

Our knowledge of bacterial autolysis is as yet far from complete, and the little information which has been acquired is confined, with very few exceptions, to the changes which take place in the protein of the bacterial cell. In some instances the investigations have not gone beyond a morphological study, and in very few cases were the changes determined by modern chemical methods.

There is considerable difference of opinion in regard to the definition of the term "autolysis." The word has been variously used to express changes in gross and microscopic appearance, loss of vitality, changes in solubility and actual changes in chemical composition. From a careful consideration of the phenomena which Salkowski originally observed and for which Jacobi first introduced this term, and in view of its extensive usage in the biochemical field, the authors would define "autolysis" as "the breaking down and solution of some of the essential chemical constituents of the cell by agencies (enzymes) originating within the cell." As bacteria are composed mainly of protoplasm it may be assumed that in ordinary autolysis the hydrolysis is chiefly of a proteolytic nature. It is for this reason that the present investigation has been concerned wholly with the changes which the proteins and related complex nitrogenous substances undergo.

The logical basis for any serious consideration of the phenomenon of autolysis must be a study of the chemical changes involved. The hydrolysis of protein or protein-like substances consists in the decomposition of the more complex molecules into simpler components. This constitutes a change in the

absolute and the relative amounts of the various forms of nitrogen. The present investigation is essentially an attempt to follow these changes by simple and practical chemical and physico-chemical methods.

METHODS AND EXPERIMENTAL PROCEDURE

The quantitative biuret test as employed by Vernon (1903) was found to be a valuable method of determining the relative amounts of protein and other closely related nitrogenous substances. Increases in amino and ammonia nitrogen were followed by the Sörensen titrations (1908). Amino nitrogen was determined also by the Van Slyke method (1911 and 1912). Observations were also made of the changes in conductivity of the autolyzing suspensions of bacteria, a procedure used by Sjöquist (1895) and later by Bayliss (1904) in their studies of digestion.

The organisms which were selected for the investigation may be placed in three distinct classes. The first is made up of those which are powerfully proteolytic, *B. subtilis*, *Erythrobacillus prodigiosus*, and related species. These are all gelatin-liquefiers. The second class is non-proteolytic and includes the colon-typhoid group. The third includes certain of the pathogenic cocci which possess little or no proteolytic power, namely *Staphylococcus aureus*, *Streptococcus pyogenes*, the pneumococcus, gonococcus and meningococcus.

The non-pathogenic organisms were grown on plain two per cent agar. Some of the pathogenic cocci required special media. A luxuriant growth of the pneumococcus, gonococcus and meningococcus was obtained by the addition of one part of ascitic fluid to two parts of three per cent agar. An even more satisfactory medium was the testicular extract agar which Hall (1916) recommends for the cultivation of the gonococcus. This medium has the advantage of not being affected by ordinary sterilization methods. The meningococcus was grown on liver extract agar in the experiments which involve amino nitrogen determinations. In one instance the pneumococcus was grown in fresh extract broth and the organisms removed by centrifuging.

Kolle flasks were employed in order to supply a maximum surface for growth. The condensation fluid was removed with a pipette and the surface allowed some time to dry, before inoculation. The inoculum was taken from young test tube cultures of the same media, and spread over the surface with a bent glass rod. The flasks were incubated for varying lengths of time, usually until the maximum growth was obtained. *Bact. coli* and the different cocci were grown at 37°C. The other cultures were incubated at 24 to 30°. The harvesting was accomplished by scraping the growth from the surface with a stiff steel wire and transferring to sterile glass-stoppered bottles. Suspensions were then made by adding sterile 0.8 per cent sodium chloride solution, or in some instances distilled water, and shaking vigorously. After filtration through sterile cotton two to five per cent of toluol was added and the bottle well shaken. After the withdrawal of a small portion of the suspension for preliminary examination, the bottles were placed in the incubator. They were shaken frequently and samples withdrawn from time to time for analysis. The different analyses were made as follows:

The biuret test

Twenty cubic centimeters of 4 per cent NaOH were measured into a Nessler tube and 2 cc. of $\frac{1}{100}$ normal $CuSO_4$ added. A measured amount (usually 0.1 to 0.5 cc.) of the supension was introduced with a sterile pipette. The amount was gauged by the intensity of color that developed. The most accurate readings are made if the test is so regulated as to give a comparatively faint color. Color comparisons were made with standard tubes to which definite but varying amounts of Witte's peptone had been added. The color strength was expressed in the terms of the percentage of Witte's peptone to which the unknown was equal in biuret-giving properties. It was found advantageous to use 0.1 per cent Witte peptone solution which (within the range of 0.1 to 2 cc.) in the standard tubes gave readable differences in 0.1 cc. gradations. These represent gradations of 0.1 mgm. of Witte's peptone. One cubic centimeter is a fair amount of the unknown to use for a test. One tenth

milligram of peptone in 1 cc. is a 0.01 per cent solution; 0.2 mgm. is a 0.02 per cent solution. It is possible, therefore, to read differences of 0.01 per cent. Slight cloudiness occasionally obscured the color to some extent. It could often be removed by centrifuging or allowing the turbidity to settle out.

The Sörensen test

Five cubic centimeters of the bacterial suspension were added to 30 cc. of distilled water. After neutralization, 5 cc. of neutral formaldehyde were added, and the resultant acidity titrated with N/20 sodium hydroxide, phenolphthalein being used as an indicator. The titrations were made immediately after the formaldehyde was thoroughly mixed with the test sample. As the results were to be used for comparative purposes only, the titrations were expressed in the number of cubic centimeters required to neutralize 100 cc. of the undiluted suspension. The amount of nitrogen in milligrams may be determined by multiplying this figure by 0.7.

Conductivity determinations

These were made with 10 cc. portions of the autolyzing material and in a conductivity cell having bright electrodes. Apparatus and methods conform with those in use in ordinary class room work. The electrodes were not platinized, owing to the possibility of a disturbing catalytic action being exerted by the finely divided platinum black. The zero point on the Wheatstone Bridge as determined by the minimum buzz in the telephone receiver was consequently not as distinct as when electrodes are platinized, but it was found that with a little practice duplicate readings could be made which checked within 2 mm. Even this slight variation was reduced by taking at least six readings within the middle third of the Wheatstone Bridge scale, employing different resistances. The determinations were made at 25°C.

The Van Slyke method for the determination of amino nitrogen

This method was found to yield valuable results when certain necessary precautions were observed. The possibilities

of variation could never be sufficiently reduced, however, to place much reliance on a single determination. At least two determinations were always made, and when these gave results differing from each other by more than 10 per cent the process was repeated until satisfactory checks were obtained. The difficulties encountered were in all probability due to the presence of bacterial cells and other suspended matter in the test material. It soon became apparent that the number of oscillations had to be regulated (240 per minute) and that more than five minutes was required to obtain a complete reaction. Ten minutes was finally adopted as the routine time. This was followed by an additional shaking by hand for one or two minutes while the motor was being used to shake the Hempel pipette. The additional gas liberated at this time was also analyzed and its volume of nitrogen added to the first determination. This gave a means of judging whether the reaction had been completed in the time allowed. Because of the extra time and labor required to obtain reliable results, this method of determining the amino nitrogen proved less practical than that of Sörensen. The original method of Van Slyke (1911, 1912) was adhered to as closely as possible, and the calculations of amino nitrogen made by reference to the tables.

EXPERIMENTAL DATA

Early in the investigation the course of autolysis was followed with the biuret and Sörensen tests. The results appeared to indicate a direct relationship between proteolytic activity and gelatin-liquefying power of bacteria, on the one hand, and autolysis on the other. Thus, *B. subtilis* suspensions underwent complete autolysis within two to three days, while *Bact. coli* showed no changes. *Ps. pyocyanea*, while slower to undergo self digestion than *B. subtilis*, gave biuret figures which approached 0. With both of these organisms the Sörensen figures rapidly increased. In the study of *Bact. coli* material no changes could be observed in the biuret or Sörensen values (see chart 1).

Following these earlier observations, the plan of study was considerably enlarged, and efforts were made to acquire as

complete evidence as possible concerning so-called "autolysis" in certain organisms selected for the purpose. As the work concerned entirely changes going on in the complex nitrogenous substances of the dead bacterial cells, the following methods were employed and found in a large measure to supplement one another and to act as checks on each other:

1. The quantitative biuret test as used and advocated by Vernon.
2. The Sörensen method for the determination of amino nitrogen.
3. The Van Slyke method for the determination of amino nitrogen.
4. The conductivity test.
5. Staining and microscopic examination of the bacterial cells.

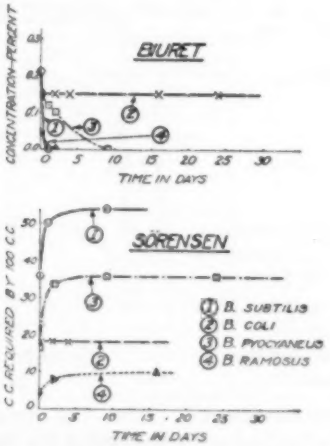

CHART 1. *B. subtilis, B. mycoides, Bact. coli,* AND *Ps. pyocyanea*

The biuret readings are expressed in percentage of Witte's peptone which will give a color of equal intensity.

The Sörensen figures are expressed in the number of cubic centimeters of $\frac{N}{10}$ NaOH required to neutralize the amino acids in 100 cc. of the solution.

Erythrobacillus prodigiosus

This organism was chosen because it readily undergoes autolysis and can be easily obtained in large quantities. The accompanying charts (charts 2 and 3) show the course of changes

observed in five different experiments by the conductivity, biuret and Van Slyke methods. Number 4 was a physiological saline solution suspension of the agar surface growths, while the others were made up in distilled water. Two strains of *E. prodigiosus* were used in these tests.

The rate of autolysis was most rapid within the first twenty-four hours, but required from ten to twenty days to be practically completed. The biuret figures continued slightly

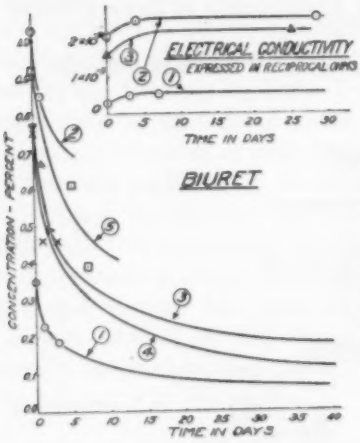

CHART 2. *Erythrobacillus prodigiosus*

Electrical conductivity is expressed in reciprocal ohms.

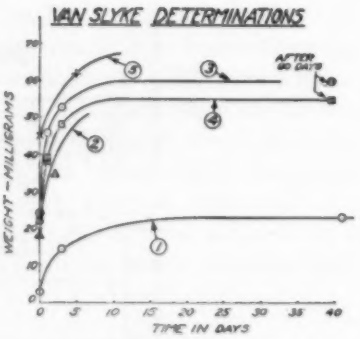

CHART 3. *Erythrobacillus prodigiosus*

The Van Slyke determinations are recorded in the number of milligrams of primary amino acids in 100 cc. of the bacterial suspension.

in the downward course even after the thirtieth day, whereas the Van Slyke values attained their maximum by the tenth day in two experiments, and by the twentieth day in a third. The other two experiments were interrupted early. The biuret and Van Slyke figures are in fairly close agreement; and both are in a large measure supported by the results of the conductivity tests. The conductivity rose during the first seven to

eight days, but remained stationary after this period. Its increase was not proportional to the amino nitrogen increase.

Bacterium coli

In the first study 14 experiments were conducted with six different strains of *Bact. coli*. No decrease in the intensity of the biuret reaction could be observed except in three instances in which only a slight change was apparent. In four of the tests only a small increase in the Sörensen figures was shown.

The biuret tests in this series were less delicate than those which followed. In the preparation of standards 0.25 per cent

TABLE 1

Showing amino nitrogen changes during incubation of suspensions of Bact. coli.

NUMBER OF EXPERIMENT	AGE OF CULTURE	ORIGINAL AMINO NITROGEN CONTENT	DURATION OF INCUBATION	FINAL AMINO NITROGEN
	hours	*mgms. per cc.*		*mgm. per cc.*
1	36	0.19	3 days	0.38
2	14	0.10	4 days	0.11
3	24	0.13	3 days	0.12
	24	0.13	3 months	0.11
4	48	0.08	8 hours	0.165
5	48	0.08	15 days	0.19
6	48	0.31	1 day	0.85
7	16	0.17	4 months	0.12
8	12	0.038	3 months	0.078
9	48	0.061	3 months	0.105

solutions of Witte's peptone were used, these giving a series of standards with 0.25 mgm. intervals between successive tubes. Each interval represents 0.025 on the ordinate of the biuret graph. Closer readings were not attempted.

Table 1 gives the results of Van Slyke determinations of amino nitrogen on 9 different suspensions. While they are not decisive, they do indicate that an increase in amino nitrogen may take place. In the three instances where no increase was shown the bacterial suspensions were prepared from cultures only twenty-four hours old or less, and the appropriate enzymes may not have had time to develop. The amino nitrogen content

was so small in these suspensions that the total gas volume seldom reached 1 cc., and hence considerable error may have been introduced into the determination, although all of the tests were run in duplicate.

In the following experiments on *Bact. coli* suspensions the biuret method was somewhat modified so as to make it more delicate. Instead of the 0.25 per cent basal solutions of Witte's peptone, 0.1 per cent strengths were employed, which furnished standards with 0.1 mgm. intervals, each interval being represented on the ordinate by 0.01.

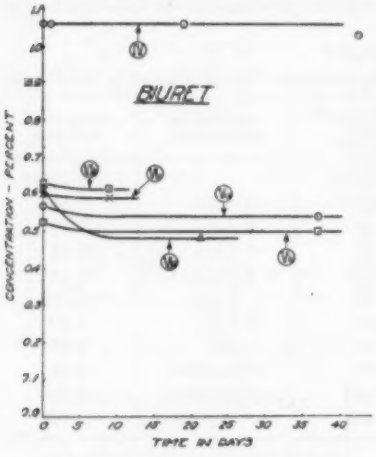

CHART 4. *Bact. coli*

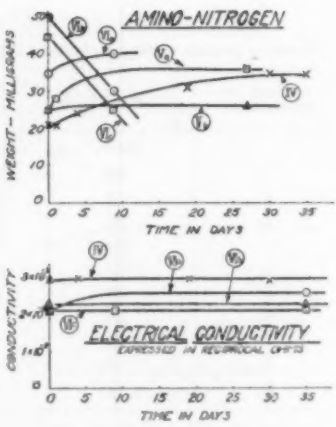

CHART 5. *Bact. coli*

Charts 4 and 5, barring curve VIa, show only a slight decrease in biuret and a corresponding increase in amino nitrogen. The biuret change in VIa was far greater than in any of the others, and is suggestive of action by enzymes other than those of *Bact. coli*, that is the presence of contaminating bacteria. It was impossible to demonstrate any contamination, though it may have existed, owing to the toluol which was added as an antiseptic, and to the inconclusive results of microscopic study.

Curves VIb and VIc represent control suspensions which were heated to 60° and 75°, respectively, for thirty minutes, before

incubation. Both show a slight decrease in biuret, and a very abrupt and pronounced decrease in amino nitrogen. This sharp drop in amino nitrogen is an anomaly, and no explanation is offered.

The conductivity of the suspensions of *Bact. coli* remained remarkably constant, except that of VIa which corresponded to the decrease in biuret. Of the three constant suspensions, however, two were heated controls.

The hydrogen ion concentrations of the different bacterial suspensions ranged from pH 7 to pH 9. Vb was brought to pH 9 by the addition of sodium carbonate, while Va remained of its own accord at about pH 8. Number VI had a hydrogen ion concentration of pH 7.

The results recorded here indicate that *Bact. coli* undergoes but slight autolysis, at the best, under conditions which are most favorable for this sort of enzymatic action.

Staphylococcus aureus and Streptococcus pyogenes

These two organisms are discussed together because they showed practically the same autolytic changes, and because the curves are given on the same chart (6). The results are of considerable interest in that the staphylococcus, which is an active gelatin liquefier, underwent but partial self digestion, and the streptococcus, which possessed no gelatin-liquefying property, whatever, elaborated enzymes which not only reduced the biuret appreciably, but brought about a marked increase in the amino nitrogen.

Four experiments were carried out with the staphylococcus, two different strains being employed. Curve 4 represents one strain, and 1, 2, and 3 the other. Curves I and II represent two experiments on one strain of streptococcus. All of these strains showed autolytic changes, although one of the strains of *Staphylococcus aureus* failed to give any evidence of this in one of the experiments (1). The suspensions were made in distilled water.

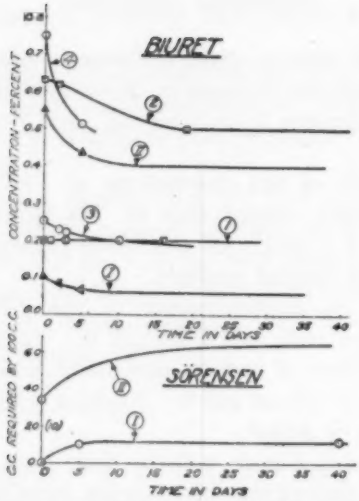

CHART 6. STAPHYLOCOCCUS AND STREPTOCOCCUS

The gonococcus

The following pages are given to a discussion of the changes taking place in certain of the pathogenic cocci, namely, the gonococcus, meningococcus and pneumococcus. Inspection of the

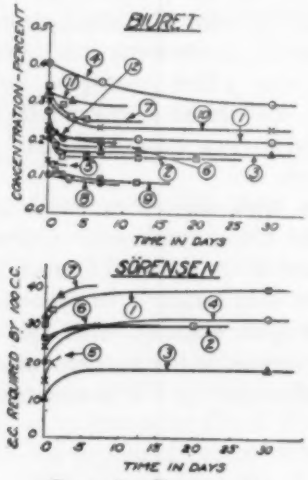

CHART 7. GONOCOCCUS

accompanying charts should leave little doubt that the process observed in these organisms is, to a certain extent at least, a true autolysis with an actual breaking down of the very complex nitrogenous substances to the amino acid stage.

Chart 7 gives the results of 12 different experiments conducted on 6 different strains of the gonococcus (2 experiments with each). Testicular extract agar and ascitic fluid agar were used, both with and without glucose, for growing the organisms. Suspensions were made in distilled water and in physiological saline solution. The curves for the biuret and Sörensen values resemble each other so closely that they need not be considered individually. It should be noted that the changes which occur develop quite rapidly. These changes in biuret and amino nitrogen were in a large measure correlated with morphological changes which could be readily observed in stained preparations of the bacterial suspensions.

The meningococcus

The meningococcus presents a picture not unlike that of the gonococcus, though there seems here to be considerably more variation in the results obtained with the different strains (see charts 8 and 9). It is of particular interest to compare the experiments in which the same strains were used, and note the near proximation of the results. Thus, 1 and 14 were the same except that 14 was kept in the refrigerator. The same strain was also used in experiments 2 and 3. Experiments 7, 8 and 12 were conducted with the same organism, and the curves both for the biuret and Sörensen values tend to be parallel. The biuret curve of 12, if shown, would coincide with that of 1. Numbers 10 and 11 are from the same strain. The biuret curve for 10, if given, would almost coincide with number 2. The organisms used in experiments 5 and 6 were obtained from the Rockefeller Institute for Medical Research, one being labeled "para-," and the other "meningococcus." The curves parallel each other almost perfectly.

These experiments were conducted over a considerable period of time and often with different, media hence the strains employed constituted about the only constant factor. From a comparison of curves 7 and 8, it appears that, within certain limits at least, variations in initial concentration do not materially affect the course of the reaction.

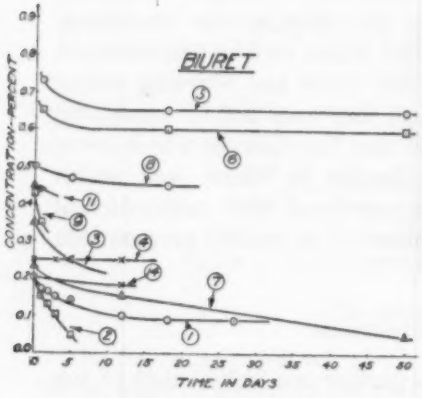

CHART 8. MENINGOCOCCUS

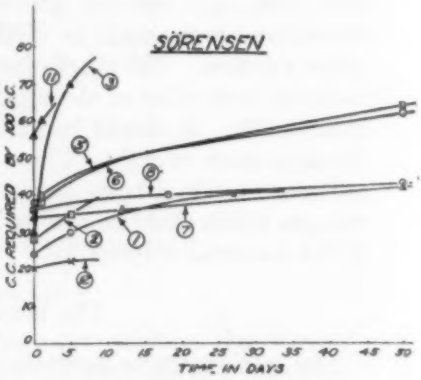

CHART 9. MENINGOCOCCUS

The pneumococcus

Thirteen experiments were conducted with 4 different strains of pneumococcus. Here again autolytic changes were evident to some extent in every instance but one (see chart 10). Number 6 was a control suspension which was heated at 70°C. to destroy the enzymes. For the sake of clearness the biuret curves for 1 and 7 were omitted from the chart. Number 1 would follow number 2 so closely as to coincide with it in the first part of its course. Experiment 7 is the one in which no changes were observed. If plotted, all of the points of this curve would be found to lie on the 0.2 ordinate. The material for this particular experiment was obtained from a sugar medium and the suspension was quite acid. It was found in certain experiments not recorded here that when emulsions of the cocci prepared from glucose agar cultures showed an acidity as high as

+1.5 to +2.5 (Fuller's scale), no autolytic changes could be observed. The other curves on these graphs present a general similarity, except Sörensen 3 and 7, in spite of the fact that they represent material grown on two different media, and for varying lengths of time, and that some of the suspensions were made in distilled water and others in physiological saline solution.

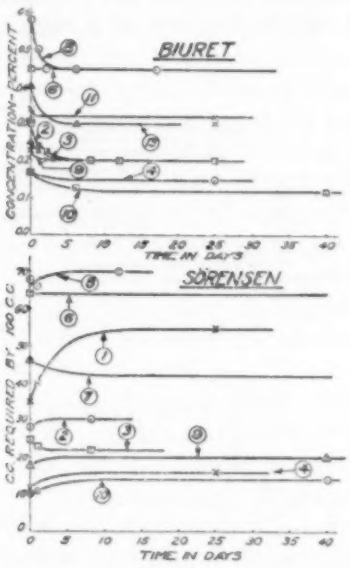

CHART 10. PNEUMOCOCCUS

Additional experiments with the pneumococcus and meningococcus

The following experiments were made possible by the generous coöperation of the Lederle Antitoxin Laboratories at Pearl River, New York. It was only by having use of the facilities of this laboratory and special assistance of a technical nature that the amounts of bacterial growth necessary for carrying out this work could be obtained.

The pneumococcus was grown in fresh extract broth, containing 0.1 per cent glucose, which had long been used for the

routine production of heavy growths of this organism. Thirty liters of this medium distributed in 3-liter bottles were inoculated from young broth cultures with the aid of a pipette, and incubated at 37°C. for fifteen hours, by which time a fairly luxuriant (about nephelometer 3) growth had developed. The broth culture was then run through a Sharpless laboratory centrifuge at a speed of 30,000 revolutions per minute. This separated out the bacterial cells in the form of a viscid paste, which was

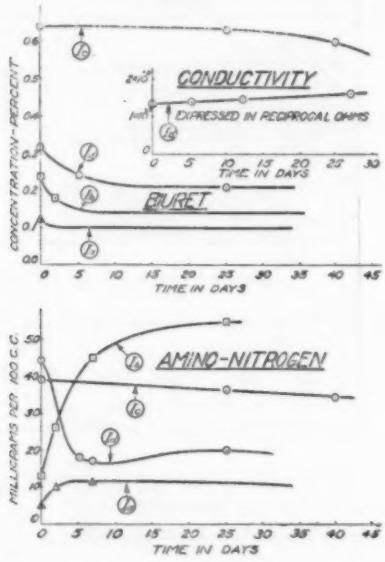

CHART 11. PNEUMOCOCCUS

scraped into a sterile, wide-mouthed, glass-stoppered bottle. About 17 grams of material were obtained which had a moisture content of 80 to 90 per cent. The bottle was packed in a freezing mixture and the contents kept near the freezing point for three days, until the experiments could be started. The bacterial paste was then divided into four parts and treated as follows:

Ia. 0.55 grams suspended in 25 cc. physiological saline
Ib. 1.0 grams suspended in 25 cc. physiological saline
Ic. 5.0 grams suspended in 40 cc. physiological saline
Id. 2.4 grams suspended in 96 cc. distilled water

The customary aseptic precautions were taken and toluol added to the suspensions. After a vigorous shaking the bottles were incubated at 37°C., with the exception of Ic which was placed in the ice box, and the autolysis allowed to proceed. The results are shown in chart 11.

A glance at the chart shows that the 3 incubated samples underwent hydrolysis to the extent of from 20 to 40 per cent of the biuret-giving substances of the bacterial cells. The amino nitrogen determinations, however, presented peculiar anomalies. In Ia and Ib there were sufficient increases to account for the hydrolysis, but Ic and Id actually showed a decrease, the discrepancy in Id being very considerable. One-third of the entire biuret-giving material disappeared without yielding any increase in amino nitrogen as estimated by these tests. Furthermore the amino nitrogen present in the beginning seemed to decrease by about half. The ratio is nearly the same for Ic, which was kept at ice box temperature, where one-sixteenth of the biuret-giving substances were lost, with an accompanying decrease in amino nitrogen of about one-eighth. No satisfactory explanation can be offered for these apparent discrepancies. That these curves represent, in the main, the actual course of the reaction is supported by the general agreement of the many readings, not all of which have been plotted on this chart. It would seem that the first reading on the amino nitrogen curve for Id is plotted a little too high. This point was obtained from the average of two out of three determinations which gave gas volumes of 1.60, 1.74 and 1.80 cc. of nitrogen, respectively.

Conductivity determinations were also made from time to time of samples taken from the test suspensions. The results are expressed in the insert in chart 11. A slight but uniform increase in conductivity was noted throughout the experiment.

To explain the anomalous behaviour of suspensions Ic and Id the hypothesis was advanced that we are concerned here with some vigorous deamidizing action whereby the amino acids lost their identity as fast as they were formed. It was too late, however, to study changes in ammonia content, but no evidence could be adduced that there had been any considerable forma-

tion of ammonia. Analysis by the Folin micro-method revealed less than 2 mgm. of ammonia nitrogen per 100 cc. of the suspension, while there were 20 mgm. of amino nitrogen to be accounted for. The deamidizing action of the autolysate was tested on a weak solution of glycocoll, but little, if any, increase in ammonia nitrogen could be detected. The long incubation of the suspension may, however, have brought about a loss of deamidizing action by this time. The hydrogen ion concentration was, at the end of the incubation period, approximately pH 7.0, which does not suggest the liberation of any considerable amount of ammonia.

It may be of some interest to note (chart 11) the close agreement of the initial determinations of the three saline suspensions. In preparing these it was attempted to give Ib a concentration twice as strong, and Ic 6 times as strong as that of Ia. While this involved careful portioning of the pasty bacterial material, which must have varied more or less in concentration in its different parts, the first determinations of both biuret and amino nitrogen show that the 3 suspensions bore the concentration relationship of 1, 2 and 6.

A liver extract agar was employed for the massive growth of meningococcus required in the following meningococcus experiment. A very luxuriant growth developed on this medium (employed in 1-liter Blake bottles) which was removed by scraping across the surface with a heavy wire bent into a loop at the end. Thirty of the Blake bottles yielded enough growth to make 11 grams of moist scrapings. This material was transferred to a sterile glass-stoppered bottle and kept in a freezing mixture for three days, or until the experiments could be started. Five grams of the viscid material were suspended in 100 cc. of sterile distilled water (Ie). The remainder was divided and one part made into a dilute (1 gram in 30 cc.) and the other into a concentrated suspension in (2 grams in 30 cc.) physiological saline solution. For the purpose of observing the effects of incubation at 37° versus low temperature, each of these two suspensions was divided into 2 parts. Five different suspensions were used, therefore, namely;

Ia, dilute (1 gram in 30 cc. of saline), at 37°C.
Ib, dilute (1 gram in 30 cc. of saline), at ice box temperature
Ic, concentrated (3 grams in 30 cc. saline), at 37°C.
Id, concentrated (3 grams in 30 cc. saline), at ice box temperature
Ie, 5 grams of the concentrated bacterial material suspended in 100 cc. of distilled water, and incubated at 37°.

The results are shown in charts 12 and 13. The biuret curves indicate an extensive autolysis of two of the incubated samples,

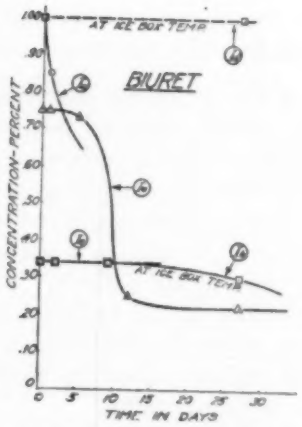

CHART 12. MENINGOCOCCUS

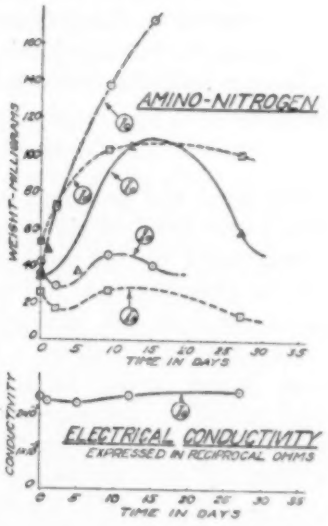

CHART 13. MENINGOCOCCUS

Ic and Ie, and but little or no change in the others. In the distilled water sample (Ie) the reduction in biuret was very slight during the first five days but subsequently progressed rapidly so that at the end of twelve days the biuret-giving substances were reduced to one-third of the original.

Owing to lack of sufficient material, duplicate amino acid determinations were made of Ie only (chart 13). All of the amino nitrogen curves have a few points of similarity, and all attain their maximum level within ten to fifteen days. Beyond this the values fall off somewhat, barring Ic of course, and, as

was observed in the pneumococcus experiments (chart 11), there appears to be some evidence of deamidization. No proof of such a process could be established, however, by further study. There is the possibility, of course, that such a deamidizing agent may have been lost from the suspension during the long and continued incubation.

Amino nitrogen curve Ie shows an apparent discrepancy. As compared with Ia and Ib, and with the biuret curve for this same suspension, the two and the five day points should be practically on the same level.

The conductivity readings for suspensions Ie show very slight changes.

GENERAL DISCUSSION

The rapid and profound autolysis of the liquefying bacteria, particularly *Erythrobacillus prodigiosus*, which could be readily demonstrated by the methods employed in this investigation, is to be expected, but that such well-known non-liquefying organisms as *Bact. coli*, the gonococcus, meningococcus and pneumococcus, should undergo a similar process, though in a much lesser degree, evoked considerable surprise in the authors. In order to satisfy themselves as to the ability of the pathogenic cocci to attack gelatin under the most favorable conditions of environment and temperature, several fruitless attempts were made to induce gelatin liquefaction in enriched media.

The enzymes involved in the autolysis of the above-mentioned non-liquefying organisms cannot but have a specific or at least a limited sphere of action. According to Rosenow (1912) the pneumococcus is able to attack the proteins of ascitic fluid, blood serum and meat extract, but we have been unable to demonstrate any proteolysis of albumin, casein or gelatin by the pneumococcus, with the use of the more delicate and specific methods employed in this work.

It has been assumed throughout the investigation that enzymes, when present, would assert themselves in a neutral or weakly alkaline medium. These experiments have as a rule been conducted, therefore, in hydrogen ion concentrations rang-

ing from pH 7.0 to 9.0. A series of tests made upon *E. prodigiosus* in different hydrogen ion concentrations showed that, although slow digestion took place at pH 4.0, the most rapid digestion occurred at pH 7.0. Rosenow claimed that the enzyme action demonstrated by him in the pneumococcus is favored in a +0.5 medium (phenolphthalein titration). Dernby (1917 and 1918), held, on the other hand, that a pH of 6.0 was the most favorable for the autolysis of animal organs.

The very slight changes which were observed in the *Bact. coli* suspensions are of particular interest and solicit further inquiry. In the first set of experiments very little enzymatic action of any sort could be demonstrated, and then in 4 of the suspensions only. In the second series (Charts 4 and 5) a slight reduction in biuret (1 per cent to 6 per cent) and an increase in amino acids were shown in every instance barring experiment VIa from which the possibility of contamination cannot be excluded. The chemical changes in these suspensions were so slight as not to be indicated at all by the conductivity tests. As the 2 heated controls showed almost the same change as the unheated test samples the "autolysis" manifestations become all the more insignificant. It is possible, however, that the heating at 60° and 75°C. may not have been sufficient to destroy the enzymes. Abbott and Gildersleeve (1903) found that heating at 100° for fifteen to thirty minutes did not always destroy certain bacterial enzymes. Meyer (1911) reported that the proteases of *Pseudomonas pyocyanea* are little affected by 15 minutes of exposure to 100°C. Wells and Corper (1912) describe lipolytic enzymes in *Mycobacterium tuberculosis* which are not entirely destroyed in thirty minutes at 100°. On the other hand, Fermi (1894) found 55 to 70° to be sufficient to destroy the proteolytic enzymes of bacteria.

The possibility of slight decreases in biuret and increases in amino nitrogen from causes other than autolysis must not be ignored. If, in the process of harvesting the bacterial growths any appreciable amount of peptone with its various biuret-giving substances should be removed from the agar medium, and if the suspended organisms elaborate enzymes which act on any

of these biuret-containing substances the results of such action would be revealed in subsequent tests. In fact, it is more than possible that the bacterial cells contain relatively simple biuret-giving polypeptides which have been absorbed from the medium or which are intermediate synthetic products occurring in the natural course of bacterial metabolism. Berman and Rettger (1918) showed that *Bact. coli* is unable to utilize the more complex or proteose fraction of Witte's peptone, but that some of the simpler biuret-containing substances in the commercial peptone, probably polypeptides, are attacked by pure culture of this organism and broken down into the simpler amino acids.

Furthermore, it is doubtful whether very slight increases in amino nitrogen need necessarily indicate any hydrolysis. We may be dealing with more or less masking of amino nitrogen by the protein, which does not allow all of the amino nitrogen to react in the Van Slyke procedure, during the early part of the incubation period. Such a protective action may to a certain extent be removed by such purely physical processes as diffusion, partial disintegration from osmotic disturbance, toluol extraction, etc. Changes of this type would render the amino acids more and more available during the course of incubation for the Van Slyke determination. Such influences scarcely seem sufficient, however, to explain the more pronounced increases in amino nitrogen noted in some of the experiments, as for example in chart 6 where the increases are shown to range from 4 to 60 per cent.

The failure to demonstrate marked changes in the suspensions of *Bact. coli* by the biuret, Sörensen, Van Slyke and conductivity methods is fully supported by direct microscopic examinations of the different suspension during the periods of observation. The individual cells at all times took the ordinary stains well and with a high degree of uniformity, and there was no evidence of morphological change, except in VIa of the second series of experiments which also differed markedly from all others in its reaction to the biuret, Van Slyke and conductivity tests, and which must be excluded from serious consideration on account of the probability of bacterial contamination.

As so few tests were made with the Staphylococcus and Streptococcus no further comment should be necessary than to state in review that both of these organisms showed distinct evidence of elaborating enzymes which have appreciable autolytic action, especially the staphylococcus.

The results obtained with the pneumococcus, gonococcus and meningococcus indicate that these organisms undergo autolytic changes as the result of their own enzyme action, and particularly the meningococcus and the gonococcus. This is further substantiated in the case of the two last mentioned organisms by the microscopic examination of stained slides in which decreased staining ability and change in cell morphology are shown during the course of the autolysis, as had been observed previously by Flexner (1907) for the meningococcus.

SUMMARY AND CONCLUSIONS

It has been possible to follow in a quantitative way the nitrogenous changes taking place in autolyzing bacterial suspensions. In this study the quantitative biuret test of Vernon and the Sörensen titration have proven of the greatest value. The Van Slyke determination of amino nitrogen has also been employed to advantage, although the material to be analyzed was not particularly suited to this method. Electrical conductivity has been found to increase during autolysis, but its increase has not been proportional to the amount of amino nitrogen formed by the hydrolysis of protein, as Bayliss (1907–8) claims to have found to be the case in the tryptic digestion of caseinogen and gelatin.

By the application of such methods it has been possible to show that:

1. Proteolytic bacteria of the type of *Erythrobacillis prodigiosus*, *Pseudomonas pyocyanea*, and *B. subtilis* autolyze rapidly.

2. *Bact. coli* undergoes slight changes which may be autolytic in nature, but which at best involve only a small part of the complex nitrogenous constituent or constituents of the cells.

3. The pathogenic cocci, pneumococcus, gonococcus and meningococcus undergo an actual autolysis with a breaking down of the protein or protein-like substances of the bacterial cells.

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BACILLUS HEMOGLOBINOPHILUS CANIS (FRIEDBERGER)

(*HEMOPHILUS CANIS EMEND.*)

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Friedberger in (1903), working in Pfeiffer's clinic reported finding in the preputial secretions of a dog a small, Gram-negative, non-motile, hemoglobinophilic bacillus which he called B. hemoglobinophilus canis. From his description it is difficult to see how he differentiated it from *H. influenzae* except that he isolated it from a dog instead of a human being. Odaira (1912), also working with Pfeiffer, compared *H. pertussis*, *H. influenzae* and *H. canis*, and found that by agglutination tests they were different. When complement fixation tests were used less specificity was shown.

Since the last pandemic of influenza much interest has been taken in the so-called hemophilic bacilli in regard to their relation to disease, their growth requirements and their biological reactions. It seemed, as advance had been made in differentiating many of these organisms by biological reactions, that *H. canis* might also be differentiated culturally from closely allied organisms. During the past year six strains have been isolated from the preputial secretions of dogs. Most of the male dogs examined had a certain amount of pus in the preputial secretions. Direct smears of this pus often showed numerous small Gram-negative bacilli and when cultured *H. canis* was usually found to be the predominating organism. Up to the present this organism has been isolated from dogs only, but it is conceivable that laboratory workers and people who handle dogs might show this bacillus accidentally in throat or nose cultures.

Morphology, staining, motility. It is a small pleomorphic, Gram-negative, non-motile rod which looks very much like *H. influenzae.*

Type of growth. On 2 per cent rabbit-blood meat-infusion agar young colonies are non-hemolytic, round, with a small granular area on top. At this time they are indistinguishable from colonies of H. influenzae but as they grow older the former become distinctly more opaque than the latter. Old cultures on blood agar slants show a luxuriant opaque growth that resembles *H. pertussis* more than *H. influenzae.* A diffuse growth occurs in the proper liquid medium.

Growth requirements. H. canis grows well on 5 per cent human blood agar. This is a distinct difference between *H. canis* and *H. influenzae* as the latter does not grow well on media containing fresh unheated human blood (Rivers, 1919). *H. influenzae* (Rivers and Poole, 1921) requires two food accessory factors, one autoclave labile, the other autoclave stable. *H. canis*, however, requires the addition of only the autoclave stable substance as an accessory factor. It will not live more than one or two generations on meat infusion agar, meat infusion agar plus ascitic fluid or 2 per cent peptone agar plus yeast extract. Successful transplants can be made indefinitely on meat infusion agar plus hematin or 2 per cent peptone agar plus hematin. It is aerobic.

Indole production. All the strains produced indole.

Nitrate reduction. All the strains reduced nitrates to nitrites.

Reaction in milk. Very little change was noticed in blood milk mixtures when inoculated with *H. canis.*

Sugar fermentations. The medium used for fermentation tests was the same as described in a previous paper. (Rivers and Kohn, 1921). All the strains formed acid without gas from glucose, fructose, galactose, mannitol, sucrose, and xylose. Neither acid nor gas was formed from maltose, lactose, dextrin, arabinose and glycerol.

Hemolysis. Red blood cells were not hemolyzed in solid or in liquid media by any of the strains.

Pathogenicity. One cubic centimeter of a twenty-four-hour culture in blood broth did not kill a white mouse when given intraperitoneally; two cubic centimeters given intraperitoneally did not kill a small guinea pig; one cubic centimeter intravenously did not kill a small rabbit.

From table 1 it can be seen that *H. canis* can be differentiated from *H. pertussis* by indole production, nitrate reduction and sugar fermentations; from *H. influenzae* by growth accessory factors and mannitol fermentation.

TABLE 1

Most important differential cultural characteristics of H. pertussis, H. influenzae and H. canis

BACTERIUM	ACCESSORY GROWTH FACTORS	INDOLE PRODUCTION	NITRATE REDUCTION	SUGAR FERMENTATIONS
H. pertussis	Old cultures require neither autoclave labile nor autoclave stable factor	Never produced	Never reduced	No sugar fermented
H. influenzae	Autoclave labile and autoclave stable factors required	May or may not be produced	Always reduced	Different sugars fermented; never mannitol
H. canis	Only autoclave stable factor required	Always produced	Always reduced	Different sugars fermented, including mannitol

CONCLUSIONS

B. hemoglobinophilus canis of Friedberger has been further described and differentiated from *H. pertussis* and *H. influenzae* by cultural methods. According to the new classification this bacillus should be called *Hemophilus canis.*

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SALT EFFECTS IN BACTERIAL GROWTH

III. SALT EFFECTS IN RELATION TO THE LAG PERIOD AND VELOCITY OF GROWTH[1]

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In a previous paper of this series (Holm and Sherman, 1921), it has been shown that certain neutral salts, in proper concentrations, accelerate the growth of *Bact. coli*, as measured by the time required to produce visible turbidity, the time required to reduce methylene blue, and the rate of acid production in the presence of a fermentable carbohydrate.

In the present work we have extended these experiments by the use of the plate count in an effort to throw more light on the mechanism of the salt action. Since our previous experiments have shown that the salt effect is magnified on the acid side of the region of optimum growth (Sherman and Holm, 1922), we have used in the present work media adjusted to a reaction of pH 5.4. All of the counts here reported represent the average of triplicate plates on extract-pepton agar incubated for three days at 33°C. Further details of the experiments are given in the appendix.

From figures 1 and 2, plotted from the data obtained in experiments 1 and 2 in which the growth of *Bact. coli* in 1 per cent pepton, 1 per cent pepton plus 0.2 M NaCl, and 1 per cent pepton plus 0.1 M Na_2SO_4 was measured, it is seen that the accelerating action of the salts is due to an increase in the velocity of growth of the organisms. In other words, the period of logarithmic growth is shortened since the number of bacteria present in the

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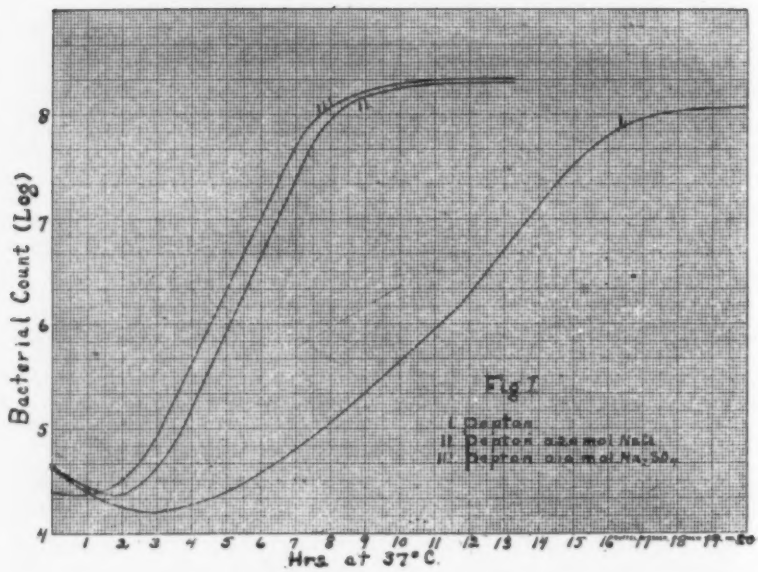
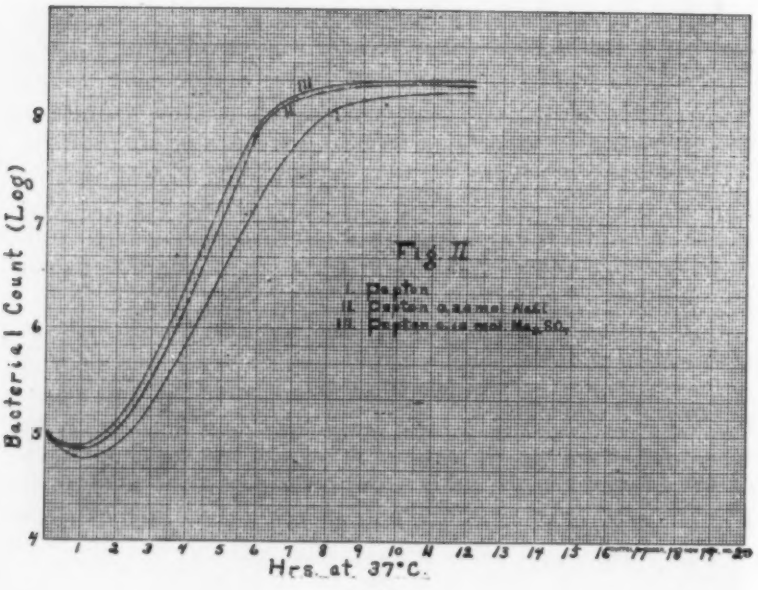

Bacterial Count (Log)
8
7
6
5
4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Hrs at 37°C.
III
II
I
Fig I
I Depton
II Depton 0.2 e mol NaCl
III Depton 0.2 e mol Na2SO4


Bacterial Count (Log)
8
7
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Hrs. at 37°C.
III
II
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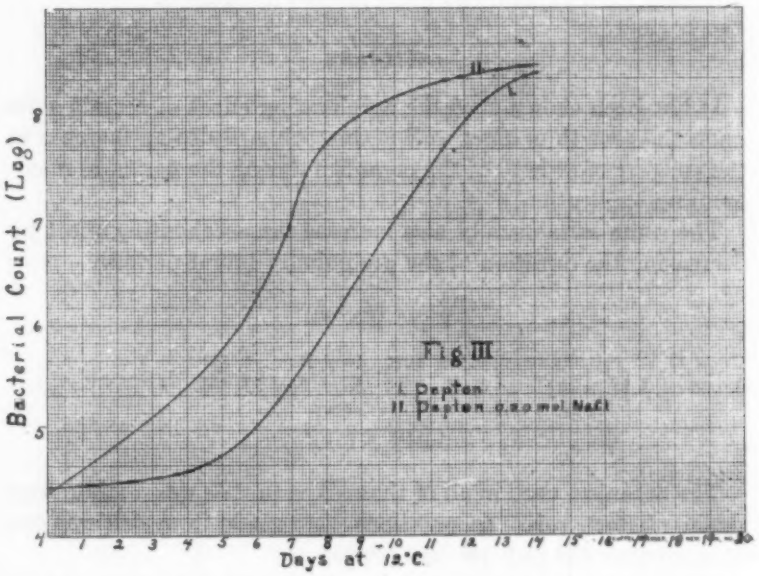
Fig II
I Depton
II Depton 0.2 e mol NaCl
III Depton 0.2 e mol Na2SO4

culture at the breaking point of the growth curve is approximately the same in all cases. It also appears that the salts have the effect of shortening the latent period previous to rapid growth. This difference is marked in the case of the sulphate (0.1 M), which appears to have a somewhat greater accelerating effect than the chloride, while in the case of the NaCl (0.2 M) the shortening effect upon the period of lag is not so definite; or may



be entirely lacking, as is indicated by the results of experiment 2.

The effect of NaCl upon the period of lag was therefore extended in experiments 3 to 6 in which plate counts were made at hourly intervals. The results of these additional experiments again show that while NaCl may or may not decrease the latent period, it increases in every case the velocity of growth during the period of active multiplication.

It was thought that it might be possible to magnify the effect of NaCl by incubation at a temperature which allowed only a

slow multiplication of the organisms. This was done in tests in which cultures were incubated at a temperature of 12°C. At this temperature the increments of growth when measured daily are about the same as those taken at hourly intervals at 37°C. The results obtained in these tests (experiments 7 and 8) show the same characteristic increase in the velocity of growth with NaCl, and also a well defined shortening of the lag period. The data from experiment 7 are plotted in figure 3.

SUMMARY

It has been shown that the accelerating effect of certain salts upon the growth of *Bact. coli* is due primarily to an increase in the velocity of growth of the organism during the period of maximum multiplication.

The same salts usually also increase the accelerating effect by decreasing the duration of the preliminary latent period.

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APPENDIX

The organism used in all of these experiments was a laboratory strain of *Bact. coli*. Inoculations were made from cultures one or more days old in 1 per cent pepton.

The media used for determining the growth rates were put up in 100-cc. amounts; all contained 1 per cent pepton, with the indicated amount of salt, and were adjusted to a reaction of pH 5.4.

In experiments 1 to 6, incubations were at 37°C. and plate counts were made at hourly intervals. Experiments 7 and 8 were conducted at 12°C. and counts made at daily intervals.

Standard extract-pepton agar was used for plating, and the plates were incubated for three days at 33°C. before counting. Triplicate plates of each dilution were made in every case.

Experiment 1

HOURS	NUMBER OF BACTERIA PER CUBIC CENTIMETER		
	Pepton	0.20 M NaCl	0.10 M Na_2SO_4 M Na_2SO_4
0	39,000	41,000	26,000
1	29,000	28,000	24,000
2	17,000	23,300	33,700
3	16,000	48,000	86,000
4	17,000	163,000	473,000
5	33,000	620,000	2,630,000
6	47,000	4,700,000	12,300,000
7	66,000	29,300,000	76,000,000
8	117,000	135,000,000	153,000,000
9	210,000	164,000,000	149,000,000
10	410,000		183,000,000
11	780,000	188,000,000	198,000,000
12	1,560,000	208,000,000	183,000,000
13	4,300,000	231,000,000	220,000,000
14	12,100,000		
16	67,000,000		
18	104,000,000		
20	121,000,000		

Experiment 2

HOURS	NUMBER OF BACTERIA PER CUBIC CENTIMETER		
	Pepton	0.20 M NaCl	0.10 M Na_2SO_4 M Na_2SO_4
0	99,000	95,000	103,000
1	58,000	79,000	78,000
2	77,000	106,000	125,000
3	300,000	181,000	471,000
4	766,000	1,750,000	2,630,000
5	3,730,000	8,500,000	16,300,000
6	17,900,000	74,000,000	74,000,000
7	49,500,000	172,000,000	168,000,000
8	109,000,000	181,000,000	191,000,000
9	149,000,000	169,000,000	201,000,000
10	148,000,000	190,000,000	209,000,000
11	162,000,000	207,000,000	214,000,000
12	175,000,000	214,000,000	215,000,000

Experiment 3

HOURS	NUMBER OF BACTERIA PER CUBIC CENTIMETER	
	Pepton	0.20 M NaCl
0	54,000	56,000
1	52,000	52,000
2	49,000	76,000
3	86,000	129,000
4	152,000	480,000
5	360,000	780,000
6	790,000	14,100,000
7	3,200,000	53,000,000

Experiment 4

HOURS	NUMBER OF BACTERIA PER CUBIC CENTIMETER	
	Pepton	0.20 M NaCl
0	56,000	57,000
1	51,000	54,000
2	59,000	59,000
3	94,000	57,000
4	148,000	139,000
5	273,000	1,120,000
6	790,000	20,600,000
7	23,700,000	105,000,000

Experiment 5

HOURS	NUMBER OF BACTERIA PER CUBIC CENTIMETER	
	Pepton	0.20 M NaCl
0	35,000	44,000
1	41,000	42,000
2	87,000	83,000
3	310,000	203,000
4	1,090,000	423,000
5	8,100,000	23,800,000
6	23,500,000	64,000,000
7	29,200,000	63,000,000

Experiment 6

HOURS	NUMBER OF BACTERIA PER CUBIC CENTIMETER	
	Pepton	0.20 M NaCl
0	126,000	119,000
1	128,000	132,000
2	175,000	188,000
3	676,000	940,000
4	2,660,000	4,440,000
5	17,800,000	85,000,000
6	24,500,000	135,000,000
7	45,200,000	205,000,000

Experiment 7

DAYS	NUMBER OF BACTERIA PER CUBIC CENTIMETER	
	Pepton	0.20 M NaCl
0	28,200	25,300
1	29,000	37,500
2	38,000	69,000
3	38,600	161,000
4	47,000	240,000
5	46,000	523,000
6	129,000	1,630,000
7	360,000	14,750,000
8	834,000	54,000,000
9	3,100,000	96,000,000
10	9,300,000	150,000,000
11	45,000,000	185,000,000
12	73,000,000	148,000,000
13	156,000,000	280,000,000
14	260,000,000	290,000,000

Experiment 8

DAYS	NUMBER OF BACTERIA PER CUBIC CENTIMETER	
	Pepton	0.20 M NaCl
0	26,800	30,000
1	29,500	36,000
2	41,000	57,000
3	36,000	182,000
4	52,000	257,000
5	53,000	340,000
6	186,000	1,480,000
7	860,000	16,600,000
8	2,020,000	54,000,000
9	7,300,000	91,000,000
10	35,000,000	160,000,000
11	56,000,000	160,000,000
12	88,000,000	186,000,000
13	165,000,000	250,000,000
14	250,000,000	270,000,000

FURTHER OBSERVATIONS ON "COLOR STANDARDS" FOR THE COLORIMETRIC DETERMINATION OF H-ION CONCENTRATION

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The following additional observations on color standards for the colorimetric determination of H-ion concentration have been made by the author since the publication of his first article on this subject (Medalia, 1920).

It will be recalled that in the first article just referred to a method was described of preparing "color standards" for the principal indicators of Clark and Lubs. This was done by using varying amounts of indicator in acid and alkaline solutions choosing such solutions as would bring out the acid and alkaline colors of the double colored indicator. Seven pairs of tubes were used; each pair containing 0.8 cc. of indicator solution in the ratio of 1:7, 2:6, 3:5, etc., increasing by 0.1 cc. in the alkaline solution up to 0.7 cc. and decreasing by 0.1 cc. in the acid solution down to 0.1 cc. These pairs of tubes were viewed by looking through them preferably in a comparator block, when placed one behind the other, i.e., superimposing the acid and alkaline colors of the indicator in the varying strengths—similar to the superimposing method used by Salm (1904) to determine the half transformation point, and by Barnett and Chapman (1918) to prepare the color standards for phenol red. The pH represented by the color of each pair of "color standards," was calibrated by using buffer mixtures of known H. I. C., prepared according to Clark and Lubs (1917).

The possibility suggested itself of making use, in addition to the seven pairs of "color standards" just referred to, of an

acid and an *alkaline* tube at either end of each set of the color standards. This would add two more colors to the range of each indicator and thus greatly enhance the usefulness of each set of color standards, more especially as regards the two *end* pairs.

Such tubes would contain each 10 cc. of the acid and alkaline solutions, respectively, as used for the particular indicator, to each of which would be added in turn 0.8 cc. of the "indicator watery solution." They would give the full acid and full alkaline colors of the particular indicator and would thus be of value in determining whether a given unknown falls within the visual or "virage" range of the particular indicator or not.

There were two questions, however, that had to be determined experimentally with reference to this:

1. Whether there would be a difference in color sufficiently marked to distinguish between such a tube, on the acid side, when compared with the first pair of the "color standards;" and the tube, on the alkaline end, when compared with the seventh pair of the set.

2. Whether any definite pH could be attributed to such tubes when studied by calibrating them with buffer mixtures of known H-ion concentration.

The first question can be answered in the affirmative: There is, according to the findings, a definite and distinct difference between the tubes on either side of the scale as compared with the respective pairs nearest to them. The solution in the tube on the acid end of the scale is the same as that described for the acid tubes of the pairs in each of the sets of "color standards" for each indicator. Similarly the tube on the alkaline end of the scale contains the solution used for the alkaline tubes of the pairs in each set.

Thus with *thymol blue* (acid range), the acid tube of this indicator is made with 0.5 volume per cent of concentrated HCl, the alkaline tube of this indicator contains 0.001 per cent concentrated HCl and to each of these is added 0.8 cc. of 0.02 per cent of the indicator watery solution.

With *brom thymol blue:* The acid tube contains 10 cc. of 0.1 per cent concentrated HCl and the alkaline tube N/200 NaOH solution.

The acid and alkaline tubes of *methyl red, brom cresol purple, brom thymol blue,* and *cresol red,* are made with 0.1 per cent concentrated HCl and N/20 NaOH, respectively. To each, of course is added 0.8 cc. of the respective indicator solution, in dilutions given in the previous article for the preparation of the color standards. The acid and alkaline tubes of *phenol red* contain 0.1 per cent HCl, and N/100 NaOH respectively,[1] and those of *thymol blue* (alkaline range) were made with 0.001 per cent concentrated HCl and N/20 NaOH respectively.

As to the second question: whether these tubes at the acid and alkaline ends of each set could be shown to represent a definite H-ion exponent, the tubes were matched against buffer mixtures of known H-ion concentration and showed the results indicated in the appropriate column of table 1.

With *methyl red:* The "alkaline tube" practically matched a pH 6.0 buffer mixture, the latter showing a very slightly more reddish tinge. With *phenol red:* The "acid tube" practically matched a pH 6.8 buffer mixture, approaching a pH 6.6 buffer mixture (there seems to be only a very slight difference in color between pH 6.6 and pH 6.8 with this indicator while, pH 6.4 of buffer and pH 6.6 are alike in color). With *cresol red:* The "acid tube" is very slightly clearer or brighter in color than a pH 7.2 buffer mixture, being practically the same otherwise. With *thymol blue* (alkaline range): The "acid tube" is slightly more yellowish in color than a pH 8 buffer mixture approaching pH 7.8 of the buffer. There is a marked difference in color between this tube and pair no. 1 of the set. The "alkaline tube" matched the pH 9.6 buffer mixture. There is a difference in color between this tube and pair no. 7, of the set.

The value of these "acid" and "alkaline" tubes, at the end of each set of "color standards" is not so much as to the *actual pH* which they represent, but rather in the fact that they allow a

[1] The N/100 NaOH was found to be the better strength to use for the alkaline tubes for the color standards of this indicator rather than N/20 NaOH as recommended in the previous article.

TABLE 1

Data for the preparation of the "color standards" and the pH which they represent

INDICATOR WATERY SOLUTION		THYMOL BLUE (ACID RANGE)	BROM PHENOL BLUE	METHYL RED	BROM CRESOL PURPLE	BROM THYMOL BLUE	PHENOL RED	CRESOL RED	THYMOL BLUE (ALKALI RANGE)
Acid tube	Alkali tube								
cc.	cc.	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*
0.8	0	1.2	3.2	4.4	5.2	6.2	6.8	7.2	8.0
0.7	0.1	1.4	3.4	4.7	5.4	6.4	7.0	7.4	8.2
0.6	0.2	1.6	3.6	4.9	5.6	6.6	7.2	7.6	8.4
0.5	0.3	1.8	3.8	5.1	5.9	6.8	7.4	7.8	8.6
0.4	0.4	2.0	4.0	5.2	6.1	7.0	7.6	8.0	8.8
0.3	0.5	2.2	4.2	5.4	6.3	7.2	7.8	8.2	9.0
0.2	0.6	2.4	4.4	5.6	6.5	7.4	8.0	8.4	9.2
0.1	0.7	2.6	4.6	5.8	6.7	7.6	8.2	8.6	9.4
0	0.8	2.8	4.8	6.0	6.8	7.8	8.4	8.8	9.6
Per cent in Indicator.* Watery solution		0.02	0.02	0.02	0.02	0.02	0.04	0.02	0.02
Per cent solution to produce alkaline color, in 10-cc. amounts		0.001 HCl	N/200 NaOH	N/20 NaOH	N/20 NaOH	N/20 NaOH	N/100 NaOH	N/20 NaOH	N/20 NaOH
Per cent solution to produce acid color, in 10-cc. amounts		0.5 HCl	0.1 HCl	0.1 HCl	0.1 HCl	0.1 HCl	0.1 HCl	0.1 HCl	0.001 HCl
Color of the "color standards"		Red through red-yellow to yellow	Yellow through pink-red to blue	Red through red-yellow to yellow	Yellow through pink to purple	Yellow through green to blue	Yellow through pink to red	Yellow through pink to red	Yellow through green to blue

* Made from alcohol 0.2 per cent stock solution, for the preparation of which see body of paper, under heading of "stock alcoholic solution."

more correct measurement of an unknown when it matches or nearly matches pair no. 1, or pair no. 7, of the "color standards."

The actual pH which the "acid" and "alkaline" tubes represent cannot be assigned to an unknown: because the unknown may be much more acid or much more alkaline than the "acid" or "alkaline" tubes at either end of the set although it does not show a different color. The value of these end tubes therefore lies in the fact that there is a distinct difference in color between the "acid tube" and the first pair, of the set on one side, and the "alkaline tube" and the last pair (pair no. 7), at the other end. The color of an unknown can be better judged when found to be between the "acid tube" and the *first pair*, or between the *last pair* and the "alkaline tube," but such judgment should be confirmed by the use of another indicator next to the one used.

TABLE 2

Clark's table giving the amounts in cubic centimeters of N/20 NaOH per decigram (0.1 gram) of indicator powder

MOLECULAR WEIGHT	INDICATOR	N/20 NaOH PER DECIGRAM
		cc.
354.17	Phenol red	5.7
669.82	Brom-phenol blue	3.0
382.17	Cresol red	5.3
540.01	Brom-cresol purple	3.7
466.30	Thymol blue	4.3
624.12	Brom-thymol blue	3.2
269.12	Methyl red	7.4

It was thought best to bring together all the data necessary for the preparation of the "color standards" with the additional "end tubes" in the form of a table, which would also show the pH values which these "color standards" represent. This will facilitate the use of the colorimetric determination by means of the "color standards" described in the previous article and the additional colors described in the present article. These data may be found in table 1.

The author's attention has been called to certain minor difficulties which various workers have experienced in using the "color standards" described in the previous article already

referred to. The most important of these was the difficulty experienced in dissolving the powdered indicators as directed for the preparation of the "stock alcoholic solution." Such difficulties can easily be overcome by using N/20 NaOH in the amounts recommended by Clark (1920) which are the molecular equivalents of each indicator, for the alcoholic stock solution (see table 2).

"STOCK ALCOHOLIC SOLUTION"

Method of preparation

The powder (say, 0.1 gram) is weighed out on an analytical balance directly in a clean, dry, heavy-walled test tube, a few drops of the N/20 NaOH is added and the powder is rubbed up with a glass rod; the rest of the required amount of the N/20 NaOH is gradually added. The dissolved indicator is now washed into a 50-cc. graduate with 95 per cent ethyl (ordinary) alcohol, and is made up to 50 cc. with the alcohol, making a 0.2 per cent "stock alcohol solution." The "indicator watery solution" is prepared from the stock alcoholic solution as already described in the previous article.

There has recently appeared an exhaustive study on "The Reaction of Culture Media" by Bunker and Schuber (1922). These authors thoroughly discuss the question of H-ion concentration with particular reference to culture media, and in referring to the author's method, mention that their "indicator watery solution" spoiled in a few days. The "indicator watery solution" has kept well in this laboratory since October, 1919 (for nearly two and one-half years), and is still in good condition. To secure this satisfactory result the author feels that it is necessary to observe a fair amount of aseptic technic. The same sterile pipette can be used for one day for the same indicator, by keeping the pipette upright in a sterile test tube.

REQUIREMENTS FOR THE PREPARATION OF "COLOR STANDARDS"

The requirements for preparing a set of "color standards" for the colorimetric determination of the H-ion concentration

of any double color indicator, which would be useful and workable are:

1. The alkaline and acid solution shall be such as will bring out the alkaline and acid colors of the indicator in a well defined way.

2. The solutions used shall be of a strength that will not fade quickly.

3. They shall be of a strength that will not bring out secondary colors.

4. The indicator solutions shall be of a strength such that when made use of to prepare the color standards in the manner described they shall produce definite, strong and unmistakable color differences between the adjacent pairs at an interval of pH 0.2.

5. The amounts of indicator used in preparing the color standards shall be measured in a way that can be correctly duplicated at any time. This can best be done by using fractions of a cubic centimeter from a graduated pipette.

6. The different colors, as represented by each pair of tubes of the color standards, shall be calibrated with buffer mixtures, of known H-ion concentration containing the same amount of the buffer mixture as is present in *each* tube of the pair and exactly the same amount of indicator solution present in both tubes of the pair.

7. The work of calibrating the "standard colors" shall be done with a comparator block having 9 holes, permitting of having on each side of the "unknown" a tube with 10 cc. of buffer mixture of known H-ion concentration containing 0.8 cc. of indicator watery solution.

When measuring an unknown solution that is turbid or colored, an extra tube of this unknown should be placed in front of each standard color in the comparator block.

8. The test tubes used for the color standards must be of clear glass and of a caliber (14 to 15 mm. inside dimensions) such as to give sufficient density of color and to be as nearly alike in diameter as possible.

All these requirements the author has tried to meet and has met in the "color standards"—as previously described.

A criticism (Gillespie, 1921) of this method of preparing color standards based on theoretical mathematical calculations has no weight, if the colors, actually prepared as described, do actually match other solutions of known pH. The calibration of each "pair" in the author's series has been made by such actual comparisons, and the pH values assigned are based upon actual visual agreements with known standards and not from hypothetical relations.

As to the criticisms brought against using these pH measurements for the study of acid production by bacteria, it is the author's point of view, that the value of being able to study from hour to hour the change produced in media by a given culture without having to disturb the sterility of the culture, and the ability to measure such change in reaction in either direction in such a simple way that can be duplicated, is beyond measure. From that point of view, it is not the question of "*how much*" acid is produced, in an abstract way, in which we are interested; but rather the change in reaction, produced, biologically, in a culture medium suited for the growth of a particular organism, in a relative way. This is of particular value if it can be done without contaminating the culture or introducing foreign elements.

It is important that the experimenter state, however, what method is used in determining acid or alkali production by bacteria, and for that matter in making use of the pH measurement for any other purpose. This will avoid confusion when the work is duplicated by others.

In concluding I wish to express my appreciation to Prof. S. C. Prescott, and Prof. J. W. M. Bunker, and Dr. M. P. Horwood of the Department of Biology and Public Health of the Institute for their helpful assistance and criticism rendered me in this work.

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THE CAUSE OF EXPLOSION IN CHOCOLATE CANDIES[1]

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During the past three years a number of instances of explosion of chocolate candies have been brought to the writer's attention. It appears that explosion in chocolates is rather a common occurrence, that it affects a considerable percentage of certain lots, and that few if any chocolate candy-makers escape the difficulty. If the chocolates are sold soon after they are made the trouble does not have time to develop, but concerns engaged in wholesale manufacture are likely to be caught at times. In case the trouble develops, the candy must be sold at a lowered price; or it is worked over into other products. In either case the economic loss is sufficiently great to merit a careful investigation of the problem.

SURVEY OF LITERATURE

An extended search of the literature has been made, but no reference dealing directly with this problem has been found. A number of investigators, notably Prescott, Stiles, and Cummins, have dealt with the sanitary aspects of the candy industry, but the explosion of chocolates was not noted.

The trade journal, Western Confectioner, July, 1920, contains a two-page article by J. P. Booker, in which he says: "After working on the problem for fourteen years, I am convinced that the bursting of chocolates is caused by the germ coli." The conclusion is said to be based upon the work of several chemists. The germ is supposed to come from water and from starch.

[1] Presented at seventy-third annual meeting, Society of American Bacteriologists, Dec., 28, 1921

DESCRIPTION OF PHENOMENON

The explosion of the chocolates occurs some time after their manufacture, usually ten days to two weeks or longer. At times the chocolate coating is merely cracked while at others it is broken into a number of fragments. If the fondant used is very moist leakage may occur. Under any circumstances the centers of the candies are exposed, the product presents an untidy appearance and deterioration occurs. As a rule the flavor is impaired, owing to the development of rancidity and off flavors.

Apparently the candy maker is unable to foretell the trouble, which does not seem to be limited to any particular type of chocolate. However, the chocolate must have a fondant center. A fondant is composed of egg white, sugar, flour, flavoring and perhaps other ingredients such as cream, butter, nuts, fruits, etc.

EXPERIMENTAL RESULTS

From the character of the phenomenon, it seemed quite probable that it was due to fermentation. Accordingly, some preliminary tests were made to discover possible fermentative organisms. Smith fermentation tubes containing glucose bouillon and a piece of meat were employed and portions of the fondant from exploded chocolates were inoculated into them under aseptic precautions; they were then incubated at 37°C. for three days or more. Many of the tubes showed extensive gas formation.

Attempts to isolate the gas formers contained in the Smith tubes were futile until anaerobic methods were employed. The anaerobe commonly present was a spore-former of the *B. sporogenes* type. Some times the organism was present in pure culture but usually other spore-formers of the *B. subtilis* type were also present. Non-sporulating bacteria were apparently absent.

Source of the gas-forming anaerobe

Obviously the anaerobe causing the fermentation might come from any one of the constituents entering into the fondant. Suspicion would attach especially to the egg white rather than

to the other ingredients. The egg used by manufacturers is the dried material secured in barrel lots. Some of this egg albumen was secured and analyzed for gas-formers with results paralleling those obtained with the fondant.

In preparing the egg albumen abundant opportunity is afforded for contamination, since the eggs used are not sterilized before breaking. The shell is frequently contaminated by hen feces in the nest.

Hen feces were next tested for anaerobes. It was necessary to heat at 80°C. for ten minutes to kill off *Bact. coli* and similar forms. Anaerobes of the *B. sporogenes* type were present in feces secured from two flocks of chickens. Thus a presumptive chain of evidence attaches blame to the egg albumen as the probable source of contamination.

Before using the egg albumen it is necessary to dissolve it by soaking in water for a number of hours. This affords an excellent opportunity for the spore formers to grow and be in condition to develop gas when the fondant is prepared and coated with chocolate. The chocolate cover would retard the absorption of air, thus facilitating the growth of the anaerobes. When the fermentation of the sugar has developed sufficient gas pressure, the chocolate explodes.

At this time the work was taken up by Miss Grace A. Hill, since appointed bacteriologist in the State College at Pullman, Wash. Miss Hill repeated and extended the work very materially, and her results were embodied as part of her master's thesis (University of Washington, 1920). She analyzed five lots of exploded chocolates secured at different times from individual firms. In all, 30 trials were made involving approximately 150 chocolates. Of these 30 trials, 17 revealed gas forming bacteria, while 13 failed to show them. One of the five lots repeatedly failed to show gas-forming bacteria.

From the above analyses Miss Hill isolated a spore-forming anaerobe producing gas in sugar bouillon. All the common sugars as well as mannitol, dulcitol and inulin were fermented with gas formation. This organism we have since identified as *B. sporogenes* by agglutination and cultural studies.

Miss Hill also isolated a second gas-forming anaerobe which apparently did not form spores and which soon died out in her cultures, thus preventing identification or further study. In our earlier and later studies this organism has not been found. Yeasts were found in some of the chocolates in one lot, but since the candies were badly broken the yeasts were assumed to be possible contaminations and were not studied further. Aerobic gas-formers of the *Bact. coli* type were never found although lactose litmus agar plates were repeatedly made from the fermentation tubes.

It was noted above that one lot of cracked chocolates never gave gas-formers when inoculated into glucose Smith tubes which were freshly boiled to drive out absorbed oxygen, and which contained a piece of meat to promote anaerobiosis. The writer has since received another lot of candies which contained cherry centers and showed cracking. When tested as before, these failed to show gas-forming bacteria. The cherries taken from the barrel also failed to show gas formers. Apparently the cracking of chocolates may at times be due to non-bacterial causes, and in the above case this cause seemed quite clearly to be rough handling by the employes.

It is possible that expansion due to increased temperature may also cause cracking, but we have no evidence for this; we know, however, that normally a chocolate slowly loses its moisture content and this would tend to prevent cracking. Apparently true explosions in chocolates are always due to the accumulation of gases formed by fermentative microörganisms.

INOCULATION EXPERIMENTS

Thus far the evidence strongly favors the conclusion that gas-forming anaerobes cause the explosion of chocolates. To make the conclusion certain, inoculation experiments were carried out. These experiments were performed in two ways. In the first trial, various kinds of chocolates were secured, small holes drilled through the coating, and a culture of *B. sporogenes* was inoculated into the fondant through this hole by means of a

heavy platinum needle. The opening was then sealed with melted chocolate, and the samples incubated at room temperature. None of the chocolates exploded when incubated for fifteen days. It seemed probable that the chocolates used had evaporated too much of their moisture content to permit growth of the organisms.

The experiment was then repeated by inoculating the culture into the fondant just before dipping. The freshly dipped and inoculated chocolates were then carried to the laboratory and incubated at room temperature as before. On the tenth day the inoculated chocolates were found exploded, while the controls remained intact after several months. The fondant consisted of egg white, sugar, butter and ground walnuts.

The experiment was repeated by adding cultures of *Bact. coli* and *Sacch. cereviceae*. The chocolates inoculated with *Bact. coli* failed to explode after prolonged incubation, but those inoculated with the yeast as well as those inoculated with *B. sporogenes* were found exploded on the tenth day. The controls remained intact. The findings indicate that the yeasts found in one of our earlier analyses were perhaps not without significance. Apparently too, more than one type of organism is capable of producing gaseous fermentation with consequent explosion of the chocolates. We have no evidence to show that the *Bact. coli* type of organism is ever causally related to this phenomenon since it was never found in exploded chocolates, and inoculation experiments were negative.

SUMMARY

1. Chocolate candies are subject to explosion due to the development of gas-forming microörganisms within them. This type of explosion should be distinguished from mere cracking due to rough handling of the candies.

2. Anaerobic bacteria of the type of *B. sporogenes* are the chief cause of explosion in chocolates, but yeasts may also cause the phenomenon. Apparently the *Bact. coli* type of organisms is not involved.

3. The source of the infection has been found in the egg albumen used, and this is probably the most prevalent source, but other materials used may obviously carry the infection at times.[2]

[2] The writer desires to acknowledge with gratitude the assistance of Miss Grace A. Hill, whose help materially advanced the work; of Miss Edna Hamilton, who assisted in part of the inoculation experiments; of Mr. Fred A. Anderson, scientific expert for one of the local candy companies, for valuable information and advice; and of all the local firms which cooperated in the work without whose aid it could not have been completed.

MICROÖRGANISMS CONCERNED IN THE OXIDATION OF SULFUR IN THE SOIL

IV. A SOLID MEDIUM FOR THE ISOLATION AND CULTIVATION OF THIOBACILLUS THIOOXIDANS[1]

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Thiobacillus thiooxidans is the organism chiefly responsible for the oxidation of sulfur in the soil, particularly when elementary sulfur has been added. This is true in ordinary soils, with the possible exception of the black alkali soils, where other organisms may also play an important part, as pointed out in the following paper. Th. thiooxidans is usually not originally present in the soil, but is introduced there artificially with the sulfur added. Certain soils themselves may harbor organisms which are able to oxidize small amounts of sulfur, to a much smaller extent however than *Th. thiooxidans*

This organism has been isolated from soil-sulfur-rock phosphate composts (Lipman, Waksman and Joffe, 1921; Waksman and Joffe, 1922) by the use of liquid media only. It can exist and reproduce under very acid reactions, since it has its optimum at a pH = 3.0 to 4.0, and can also grow at as low a pH as 1.0. It derives its energy from the oxidation of inorganic, elementary sulfur, and its carbon from the CO_2 of the atmosphere, while its nitrogen need is obtained from ammonium salts or nitrates and its mineral need from traces of potassium, magnesium and iron salts and phosphates. By the use of media containing such substances, and by employing high dilutions the organism was isolated in pure culture. The purity of the culture was established by the following facts: the organism grew readily upon the inorganic liquid media, giving always the same uniform turbidity;

[1] Paper no. 84 of the Journal Series, New Jersey Agricultural Experiment Station, Department of Soil Chemistry and Bacteriology.

it did not grow in bouillon, on nutrient agar, or other common organic and inorganic media, favorable for the cultivation of bacteria and fungi; it gave a uniform microscopic picture, both in unstained and stained preparations. The culture was kept on liquid media, for over one and one-half years by transferring it to fresh lots of media every one to four weeks. All attempts to develop a solid medium for the cultivation of the organism failed until December of last year.

A detailed study of the media commonly used for the cultivation of colorless sulfur bacteria not accumulating sulfur within their cells is given elsewhere (Waksman, 1922). The two liquid media best adapted for the isolation and cultivation of *Thiobacillus thiooxidans* have the following composition:

I		II	
$(NH_4)_2SO_4$	0.2 gram	$(NH_4)_2SO_4$	0.2 gram
$MgSO_4 \cdot 7H_2O$	0.5 gram	$MgSO_4 \cdot 7H_2O$	0.5 gram
KH_2PO_4	3.0 grams	KH_2PO_4	1.0 gram
$CaCl_2$	0.25 gram	Re-precipitated $Ca_3(PO_4)_2$	2.5 grams
Elementary, powdered sulfur	10 grams	Sulfur	10.0 grams
Distilled water	1000 cc.	$H_3PO_4\left(\frac{M}{1}\right)$ to adjust reaction to pH = 3.0	
		Distilled water	1000 cc.

The sulfur in both media and the Ca_3PO_4 in medium II are weighed out separately in the individual flasks into which the media are distributed (100-cc. portions are usually placed in 250-cc. flasks). The media are sterilized for thirty minutes, in flowing steam; on three consecutive days.

When a flask with one of these two media is inoculated from a fresh vigorous culture (seven to fourteen days old) of the *Th. thiooxidans* growth will be manifested by a uniform turbidity, without any pellicle formation, within four to five days, at 25 to 30°C., the culture becoming very turbid in seven to eight days. The same phenomenon is observed when the medium is inoculated with a little soil containing the organisms, only the length of time required for development is sometimes a little longer, depending upon the abundance of the organism in the soil, condition of soil, etc. By using the dilution method, even the

approximate number of the organisms in the soil can be estimated. The culture obtained on the two media is practically pure, due to the fact that very few other organisms would develop under these conditions. However, both for the purpose of establishing the purity of the culture and for the better characterization of the organism, the development of a solid medium was extremely desirable.

Among the various solid media tried, without any success, for the cultivation of *Th. thiooxidans* was Beijerinck's thiosulfate medium (1904) upon which *Th. thioparus* grew readily. Although *Th. thiooxidans* can utilize thiosulfate as a source of energy, it did not grow on this medium due to the extreme alkalinity of that medium and to the lack of a calcium salt. When the $NaHCO_3$ is eliminated from this medium, the dibasic potassium salt changed to the monobasic form and when calcium chloride is added, a solid medium favorable for the growth of *Th. thiooxidans* is obtained.

The composition of the medium is as follows:

$Na_2S_2O_3 \cdot 5H_2O$	5.0	grams
KH_2PO_4	3.0	grams
NH_4Cl	0.1	gram
$MgCl_2$	0.1	gram
$CaCl_2$	0.25	gram
Agar	20.0	grams
Distilled water	1000	cc.

The medium is prepared as usual and sterilized at 15 pounds pressure for fifteen minutes. Plates and slants are inoculated from a vigorous liquid culture and incubated at 25 to 30°C. Growth appears in five to six days in the form of minute straw yellow to cream-colored colonies. Under the microscope, each colony is surrounded with crystals of gypsum due to the action of the sulfuric acid, formed from the oxidation of the thiosulfate, upon the $CaCl_2$. This phenomenon is particularly prominent in media containing tri-calcium phosphate in place of the chloride; a clear zone is formed around each colony, due to the disappearance of the insoluble calcium salt.

Th. thioparus practically makes no growth on this medium, due to its acid reaction. In the original Beijerinck medium, however, containing dibasic phosphate and $NaHCO_3$, *Th. thiooxidans* makes no growth, while *Th. thioparus* grows in the form of minute pinpoint colonies covered with sulfur, which is separated from the thiosulfate by the action of the latter organism. The *Th. thiooxidans* separates no sulfur, oxidizing the thiosulfate completely to sulfate; *Th. thioparus* oxidizes the thiosulfate to sulfate, persulfates and elementary sulfur.

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MICROÖRGANISMS CONCERNED IN THE OXIDATION OF SULFUR IN THE SOIL

V. BACTERIA OXIDIZING SULFUR UNDER ACID AND ALKALINE CONDITIONS[1]

SELMAN A. WAKSMAN

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A detailed description of media and methods of isolation of sulfur bacteria concerned in the oxidation of sulfur in the soil has been given in the previous two papers of this series (Waksman, 1922 a, 1922 b).

The work reported in this paper is limited to two liquid (nos. 8 and 6) and two solid media (nos. 9 and 10). Medium 8 consists of 5 grams $Na_2S_2O_3$, 0.1 gram NH_4Cl, 0.1 gram $MgCl_2$, 0.2 gram Na_2HPO_4 (or K_2HPO_4), 1 gram $NaHCO_3$ and 10 grams $CaCO_3$ or 0.25 gram $CaCl_2$ dissolved in 1000 cc. of tap water. This is only a slight modification of the original Beijerinck (1904) medium. Medium 9 is the same as 8, but with 2 per cent of agar added. These media are sterilized, at 15 pounds pressure, for fifteen minutes; the thiosulfate and carbonate are sterilized, in the case of the liquid media, separately, then added by means of sterile pipettes. The initial reaction of these media is about pH = 8.6 to 9.6. Medium 6 consists of 0.2 gram $(NH_4)_2SO_4$, 0.5 gram $MgSO_4$, 0.25 gram $CaCl_2$, 3 grams KH_2PO_4, trace of $FeSO_4$ and 10 grams of sulfur in 1000 cc. of distilled water. This medium is sterilized in flowing steam, for thirty minutes on three consecutive days. Its initial reaction is pH = 4.0 to 4.8. Medium 10 consists of 5 grams $Na_2S_2O_3 \cdot 5H_2O$, 0.1 grams NH_4Cl, 0.1 gram $MgCl_2$, 0.25 gram $CaCl_2$ (or 10 grams $Ca_4(PO_4)_2$), 3 grams KH_2PO_4, 20 grams of agar in 1000

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cc. of distilled water. This medium is sterilized in the autoclave, at 15 pounds for fifteen minutes.

When a small particle of soil is added to media 6 and 9, growth may or may not take place according to the nature of the soil, the two media behaving in a distinctly different way. Medium 6 is acid in reaction and contains elementary sulfur as the only source of energy; only those organisms which can oxidize elementary sulfur and can live at a pH of 4.0 and less will develop in this medium. Such organisms are not usually present in common cultivated soils, unless the soils have had previous applications of elementary sulfur. The organism developing on this medium is the *Th. thiooxidans* Waksman and Joffe (1922) described in detail elsewhere. This organism is not universally present in the soil, but is present in soil mixed with powdered sulfur, as around the sulfur mines, etc. This is the reason why the organism was originally isolated from composts of sulfur with soil or sulfur, rock phosphate and soil. This organism oxidizes sulfur, in pure culture, quantitatively to sulfuric acid; it also oxidizes thiosulfate, without the separation of elementary sulfur, with active acid formation. The growth of this organism on the liquid media is quite characteristic: it forms, in four to five days, at 25°C., a turbidity throughout the medium, without any pellicle formation. In the presence of calcium salts, crystals of gypsum soon appear, both on the bottom of the flask and hanging down from the particles of sulfur. The sulfur floating on the surface of the medium drops to the bottom. When transferred to medium 10, minute pale to pale-yellow to dirty-yellow (or light cream colored) colonies appear in five to six days. In the presence of insoluble calcium salts, like the carbonate and the phosphate, a colorless halo is rapidly formed around each colony on the plate, due to the action of the sulfuric acid formed from the oxidation of the thiosulfate upon the insoluble calcium salts. A ring of gypsum crystals is formed around each colony.

Medium 8 behaves differently; organisms that can thrive at distinctly alkaline reactions and that can oxidize thiosulfate will develop on this medium. Such organisms are universally dis-

tributed in the soil. Whether these organism form a group of closely related strains or whether they form a group of organisms differing in morphological and physiological characteristics still remains to be seen. The characteristic growth of this group of organisms is as follows: when a piece of soil is added to medium 8 (better growth will take place, when the medium is not sterilized), and flasks incubated at 25°C., growth will appear in forty-eight to seventy-two hours. This growth consists of a pale colored pellicle formed on the surface of the medium and on the glass of the flask; the medium does not become turbid, unless *Th. thiooxidans* is also present in the soil. An abundant separation of the sulfur from the thiosulfate takes place, so that the surface pellicle becomes sulfur-yellow. The thiosulfate rapidly disappears with the formation of sulfates, persulfates and elementary sulfur. If more thiosulfate is added to the flask, it will be further transformed, so that 5 grams of thiosulfate may be transformed in a 100-cc. culture in two to three weeks; this will take place thus rapidly, however, only when a proportionate amount of $NaHCO_3$ is added with the thiosulfate (1:5). The rôle of the bicarbonate consists in supplying the base to the formation of the sulfate and keeping the medium from becoming acid. Whether the bicarbonate may also serve, at least partly, as a source of carbon, is still unknown; however, it does not do so for *Th. thiooxidans*. The reaction of the medium becomes only slightly acid. Where intense acid formation accompanied by the breaking up of the sulfur pellicle has been found, *Th. thiooxidans* has invariably been demonstrated to be present. When a streak is made from the culture in medium 8 upon a plate containing cooled medium 9, minute colonies will rapidly develop, characterized by an abundant separation of sulfur, which covers the whole colony. No clear zone, or a mere trace of it, is formed around the colony, unless *Th. thiooxidans* is also present. This organism thus developing on medium 8 is closely related in its physiological characteristics to the *Th. thioparus* of Beijerinck, which was demonstrated by Gehring (1915) to be universally distributed in the soil. This organism will be called *Thiobacillus B*. This organism can be cultivated very readily,

but rapidly deteriorates on artificial culture media. Its absolute purity, particularly as far as contamination with *Th. thiooxidans* or a modified strain of this organism was not established as yet.

When sulfur is added to a neutral or acid soil, it is rapidly oxidized by *Th. thiooxidans* when present or introduced. When sulfur is added to an alkali soil, particularly a black alkali soil, the oxidation is very slow, even in the presence of *Th. thiooxidans*. However, after a little time, the sulfur is oxidized. When some of this black alkali soil, to which sulfur has been added, is introduced into flasks with media 6 and 8, an excellent growth will take place in the latter; while, in the former, growth will take place only if the oxidation is at an advanced stage. A series of studies has led the author to conclude that, while, in acid soils, the sulfur is oxidized primarily by *Th. thiooxidans*, in alkali soils *Thiobacillus B.* also takes an active part in the process. This part consists either in a direct oxidation of the sulfur, whereby the reaction of the soil is made sufficiently acid so that *Th. thiooxidans* (if present) may oxidize the sulfur further, or by a certain modification of the sulfur, so that it is more readily acted upon by *Th. thiooxidans* under alkaline conditions. The chemistry of the process of sulfur oxidation both in acid and alkali soils by the two organisms has been reported in detail elsewhere (Waksman and Joffe, 1921, Waksman, 1922 c).

The following three tables show clearly the relation between initial reaction, source of sulfur and sulfur oxidation.

The following tables definitely establish the fact that *Th. thiooxidans* primarily oxidizes elementary sulfur under acid conditions. The possibility that it may oxidize elementary sulfur in the soil, under alkaline conditions is thereby not excluded, particularly in the presence of other organisms. It also oxidizes thiosulfate with an intense acid formation. *Thiobacillus B.* oxidizes very little elementary sulfur in the soil or in solution, but the fact that it can act upon sulfur under highly alkaline conditions suggests the possibility of the associative action of the two organisms in changing the reaction of black alkali soil to which sulfur has been added. *Thiobacillus B.* oxidizes thiosulfate

TABLE 1

The oxidation of elementary sulfur at different reactions

Medium 6 without the KH_2PO_4 used as a basis

COMPOSITION	CULTURE	pH VALUE		
		Control	6 days	21 days
(A) 3 grams KH_2PO_4	Th. thiooxidans	4.2	1.6	1.2
(B) 1½ grams KH_2PO_4 + 1½ grams K_2HPO_4	Th. thiooxidans	5.4	3.0	1.2
(C) 3 grams K_2HPO_4	Th. thiooxidans	6.0	5.4	1.4
(D) 3 grams K_2HPO_4 + 10 grams $CaCO_3$	Th. thiooxidans	8.0	8.0	8.0
(A)	Thiobacillus B.	4.2	3.8	3.6
(B)	Thiobacillus B.	5.4	3.8	3.8
(C)	Thiobacillus B.	6.0	4.0	3.8
(D)	Thiobacillus B.	8.0	8.0	8.0
(A)	Th. thiooxidans + Th. B.	4.2	2.0	1.2
(B)	Th. thiooxidans + Th. B.	5.4	2.2	1.4
(C)	Th. thiooxidans + Th. B.	6.0	1.8	1.4
(D)	Th. thiooxidans + Th. B.	8.0	8.0	6.4
(A)	Ordinary soil	4.2	4.2	4.2
(B)	Ordinary soil	5.4	5.4	5.2
(C)	Ordinary soil	6.0	6.0	5.4
(D)	Ordinary soil	8.0	8.0	7.6
(A)	Acid compost (soil + rock phosphate + sulfur)	4.2	2.6	1.4
		5.4	3.4	1.4
(B)	Acid compost			
(C)	Acid compost	6.0	5.0	1.8
(D)	Acid compost	8.0	8.0	7.6
(A)	Alkali compost (alkali soil + sulfur)	4.2	4.2	4.2
		5.4	3.8	3.8
(B)	Alkali compost			
(C)	Alkali compost	6.0	4.6	4.4
(D)	Alkali compost	8.0	8.0	6.4

rapidly, with an abundant separation of sulfur. This separation of sulfur from the thiosulfate as a result of the action of this organism indicates its rather weak sulfur-oxidizing power. When *Th. thiooxidans* is also present, the pellicle is either not formed at all, especially when the medium is acid, or, in alkaline

TABLE 2

The oxidation of thiosulfate at different reactions

Medium 8 used as a basis, without any carbonate

COMPOSITION	CULTURE	pH VALUE		
		Control	6 days	21 days
(A) 3 grams KH_2PO_4	Th. thiooxidans	5.4	4.4	1.4
(B) Medium 8, without carbonate	Th. thiooxidans	6.2	5.0	1.6
(C) 1 gram $NaHCO_3$	Th. thiooxidans	8.8	8.8	8.8
(D) 1 gram $NaHCO_3$ + 10 grams $CaCO_3$	Th. thiooxidans	9.4	9.4	9.4
(A)	Thiobacillus B.	5.4	5.4	5.4
(B)	Thiobacillus B.	6.2	6.2	6.0
(C)	Thiobacillus B.	8.8	6.2	6.4
(D)	Thiobacillus B.	9.4	6.4	7.0
(A)	Th. thiooxidans + Th. B.	5.4	4.8	1.4
(B)	Th. thiooxidans + Th. B.	6.2	5.2	1.4
(C)	Th. thiooxidans + Th. B.	8.8	5.2	1.6
(D)	Th. thiooxidans + Th. B.	9.4	5.8	6.6
(A)	Ordinary soil	5.4	5.4	5.0
(B)	Ordinary soil	6.2	6.2	5.6
(C)	Ordinary soil	8.8	8.0	6.8
(D)	Ordinary soil	9.4	9.4	8.0
(A)	Acid compost	5.4	5.4	5.0
(B)	Acid compost	6.2	6.2	5.4
(C)	Acid compost	8.8	8.4	6.0
(D)	Acid compost	9.4	9.4	8.0
(A)	Alkali compost	5.4	5.2	5.0
(B)	Alkali compost	6.2	6.4	5.6
(C)	Alkali compost	8.8	8.6	5.8
(D)	Alkali compost	9.4	9.4	7.6

media, it is rapidly broken up as a result of the rapid oxidation of the sulfur to sulfuric acid by *Th. thiooxidans*.

In the study of ordinary cultivated soil, the presence of *Thiobacillus B.* or organisms closely related in their metabolism is easily demonstrated. The acid compost, which is a mixture of sulfur (5 per cent), rock phosphate (15 per cent) and soil,

inoculated with crude cultures of *Th. thiooxidans* has become so acid due to long incubation (one year) that only the latter organism survives. If *Thiobacillus B.* was originally in the compost, it has died out, due to the intense acidity. The alkali compost consisting of black alkali, to which 0.5 per cent of sulfur has been added and incubated for 6 months, definitely indicates the presence of the second organism.

The sulfide (table 3) was oxidized to some extent by both organisms, and particularly by a mixture of the two organisms. This is explained by the fact that the sulfur produced from the sulfide by the action of *Th. B.* is rapidly oxidized to sulfuric acid

TABLE 3

The oxidation of K_2S by microörganisms

Medium 8 used as a basis, 0.5 per cent K_2S in place of the thiosulfate

CULTURE	pH VALUE			SULFATES, MILLIGRAMS OF S IN 100 CC.
	Control	6 days	21 days	
Th. thiooxidans*	7.5	7.5	7.5	14.11
Thiobacillus B.	7.5	5.8	4.5	43.84
Th. thiooxidans + Thiobacillus B.	7.5	5.2	1.4	90.42

* In some cases, particularly at lower pH values, the oxidation of the sulfide by the *Th. thiooxidans* was much more extensive.

by *Th. thiooxidans*. This is well demonstrated by the column of sulfates formed. A detailed study of the morphology of these two organisms will form the subject of a succeeding paper.

SUMMARY

1. At least two organisms can be shown to take part in the oxidation of sulfur in the soil; while, in acid soils, *Th. thiooxidans* will rapidly oxidize the elementary sulfur, in alkali soils, particularly black alkali soils, still another organism, *Thiobacillus B.*, similar to *Th. thioparus* of Beijerinck, takes an active part in this process.

2. *Th. thiooxidans* is usually not present in common cultivated soils, but is found abundantly in soils previously treated with sulfur. *Thiobacillus B.* is commonly present in cultivated soils.

3. By the interaction of the two organisms, sulfur can be oxidized at reactions ranging from pH = 9.8 to 1.0.

TAXONOMIC POSITION OF THE COLORLESS SULFUR OXIDIZING ORGANISMS NOT ACCUMULATING SULFUR WITHIN THEIR CELLS

The colorless organisms oxidizing sulfur and sulfur compounds and not accumulating sulfur within their cells have been classified by Beijerinck (1904) under the genus Thiobacillus. Orla-Jensen (1909) properly indicated the relationships of these organisms both in their morphology and physiology to the nitrifying bacteria, methane, hydrogen and other autotrophic organisms, and suggested the generic name Sulfomonas in place of Thiobacillus. In view of the fact that the Committee on Characterization and Classification of the S. A. B. (Winslow et al.,1920) has adapted several of the new generic names suggested by Orla-Jensen for the members of the Nitrobactereae, the generic name Sulfomonas should be used rather than that of Thiobacillus, and this genus of sulfur oxidizing bacteria should be classified with the Nitrobactereae. This genus will now consists of three species:

Sulfomonas thioparus (syn. *Thiobacillus thioparus* Beijerinck, Orla-Jensen 1909, p. 314).

Sulfomonas denitrificans (syn. *Thiobacillus denitrificans* Beijerinck, Orla-Jensen 1909, p. 314).

Sulfomonas thiooxidans (syn. *Thiobacillus thiooxidans*, Waksman and Joffe).

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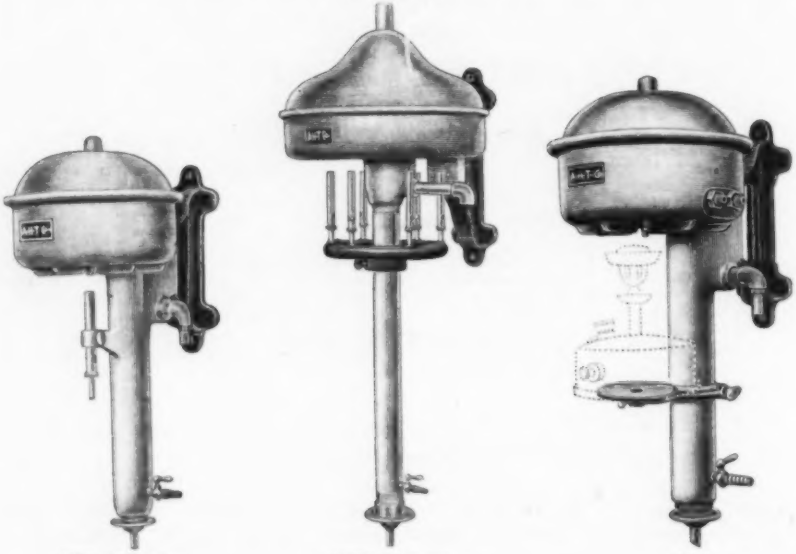

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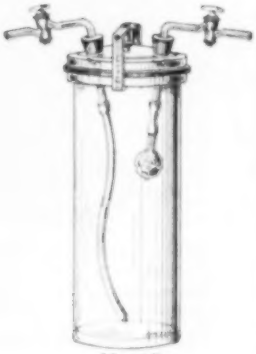

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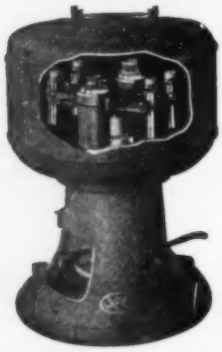

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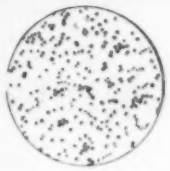

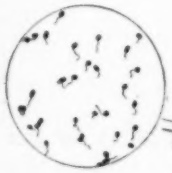

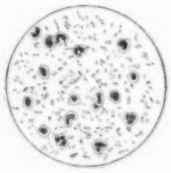

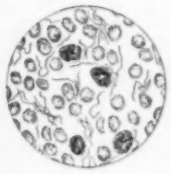

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